PHILIPPINE JOURNAL OF SCIENCE

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OUR COVER. The cover photo, taken from the first manuscript published in this issue, shows a leafy branch of *Pittosporum Resiniferum Hemsl.* with fruits.

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DEVELOPMENT OF THE FEMALE GAMETOPHYTE OF PITTOSPORUM RESINIFERUM HEMSL.* (PITTOSPORACEAE)

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ABSTRACT

The anatomy of the megaspore and the process of megasporogenesis and megagametogenesis in Pittosporum resiniferum Hemsl. is described from sections processed using a modified paraffin technique. The ovary is superior and bicarpellate. The ovule is unitegmic, tenuinucellate and ana-campylotropous. The archesporial cell functioned directly as the megaspore mother cell. The first meiotic division of the megaspore mother cell gave rise to the dyads and meiosis II to linear megaspore tetrads. The megaspore towards the chalazal end was functional while the three megaspores at the micropylar end degenerated. The functional megaspore divided mitotically three times and gave rise to the eight-nucleated female gametophyte. Development of the female gametophyte conforms to the Polygonum type and is Monosporic.

INTRODUCTION

Pittosporum resiniferum Hemsl. is a species that has been identified as a promising source of energy or as a material for petroleum-based products. It is also called *"hanga,"* but a more popular name is *"petroleum nut"* because of the resemblance of the odor of the fruit oil with that of petroleum and its property to burn brilliantly (West and Brown, 1921).

"Petroleum nut" was reported by Bacon (1909) as a source of hydrocarbon, and is commonly used for lighting purposes. Some mountain people in Palawan used the fresh fruits for fuel of their torch light. This practice was also used by the Japanese soldiers in the Philippines during World War II.

Chemical analysis of the oil showed that it contains a dihydroterpene ($C_{10}H_{18}$), a medicinal and perfumery compound, and a normal heptane (C_7H_{16}), a component of gaso-

* Part of the senior author's dissertation paper

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line (Veracion and Costales, 1981). Studies on its fuel properties showed that it is quite comparable with that of gasoline (Asia Oil Co., Ltd. 1982). Histochemical studies showed that the plant contains alkaloids, glycosides, organic acids, resins and lignins which may be responsible for its medicinal values. Other studies showed anti-bacterial property from extracts of the leaves, stem and bark (Valenzuela, 1949; Quisumbing, 1951; and Fernando, 1988).

P. resiniferum is an endemic species and is not very abundant, although it is believed to be widely distributed in the country. It is found on high mountain ridges and forested areas from Bontoc to Sorsogon, Mindoro and Cantanduanes and particularly in the Cordillera mountains and Benguet (Zamora and Tolentino, 1991). It commonly grows as an epiphyte attaching itself to bigger trees or as an independent plant. The leaves are crowded toward the ends of the branchlets and are spirally-whorled, leathery, smooth, oblanceolate and pointed at both ends with reticulate venation. The fragrant white flowers have five oblong-shaped petals and five sepals. They are regular, perfect, polypetalous, gamosepalous, short-pedicelled and glabrous. They are borne in clusters on mature stem (cauliflory). The fruit is ellipsoid, green and becomes yellow to orange when ripe (Fig. 1).

This study aims to describe the cytohistological structure and development of the megasporangium, megaspore, megasporogenesis and megagametogenesis of the female gametophyte.

MATERIALS AND METHODS

The plant material used in this study were the flowers in various stages of development. These were collected periodically from wild populations and cultivated plants in Pacdal, Baguio City, Mt. Sto. Tomas in Benguet, Bureau of Plant Industry Economic Garden in Los Banos, Laguna, and from the Bureau of Plant Industry Research Station in Luisiana, Laguna.

Paraffin method as modified by Zamora (1992)

The ovaries were dissected out of the flowers, and the ovules were isolated. A total of 845 ovules and 2,028 seeds were sectioned and processed as follows: (1) killed and fixed in FAA solution for 48 h (formalin, alcohol, acetic acid, 9:1:1), (2) washed in running water till there was no trace of acid, (3) dehydrated through the ethanol (EtOH) series (30%, 50%, 70%, 85%, 95%, 100% (I), 100% (II) for 1 h each), (4) cleared through the xylene-alcohol series (25%, 50%, 75%, 100% (I), 100% (II) for 1 h each), (5) infiltrated with soft paraffin, (50-52 °C melting point) medium paraffin (54-56 °C melting point) and hard paraffin (58-60°C melting point) for 12 h each in the oven at 60 °C, (6) embedded in hard paraffin (Merck), (7) trimmed and cut in an American Optical (AO) rotary microtome at 8-10 um, (8) affixed on glass slides previously coated in Mayer's adhesive, (9) passed through EtOH - xylol series, (100% (I), 100% (II) xylene; 100% (I), 100% (II), 95%, 85%,

70% EtOH, for 3 min each), (10) stained with 1% safranin O (safranin O dissolved in 50% EtOH) for 24 h, washed with water, then passed through the EtOH series, (50%, 70%, 95% EtOH) and counterstained with 0.5% fast green FCF (FCF dissolved in 95% EtOH) for 3 min, and finally (11) mounted in Canada balsam. Suitable materials were photomicrographed using phase contrast optics of the BH-2 Olympus research microscope.

Interpretative line drawings of appropriate serial sections were drawn to show the proper orientation of the cells of the female gametophyte. Each of the serial sections was traced and then overlaid on each other to get a reconstruction of the arrangement of the cells.

RESULTS

The Megasporangium

The ovary is superior, bicarpellate, syncarpous and bilocular. Each locule contained two ana-campylotropous, unitegmic ovules borne in an axial placenta. A dome-shaped ovule-primordium developed from the placenta of the young ovary. The ovule-primordium consists of a mass of parenchyma cells bounded by a layer of epidermis. Continuing proliferation of the mass of cells and the expansion of the epidermis by anticlinal divisions gradually lifted the whole ovule-primordium from the placental base. The initial cells of the single integument differentiated from the epidermal cells of the ovule-primordium. Simultaneous with this development was the elongation and periclinal division of the epidermal cells. This was the first indication of the curvature of the ovule which continued on one side and finally became ana-campylotropous. The ovule curved continuously and became completely inverted so that the micropyle and hilum came to lie very close to each other. It is tenuinucellate since the megaspore mother cell lies directly below the epidermis and no parietal cells differentiated . This tenuinucellate form of the nucellus is short and the primordia of the integument arose near its apex. The nucellus is surrounded by the integument which is 2-3 cells thick and became 5-6 cells thick at maturity. The point at which the integument meets is the micropyle. The ovule is attached to the placenta through the funiculus. The young carpel is pubescent with multicellular trichomes .

Megasporogenesis and The Female Gametophyte

A single, hypodermal archesporial cell functioned directly as the megaspore mother cell. The megaspore mother cell was distinct from the other cells due to its large size, dense cytoplasm, and a more prominent nucleus (Figs. 2, 3). The megaspore mother cell enlarged and elongated before dividing meiotically (Fig. 4). After meiosis I, the megaspore mother cell gave rise to two dyad megaspores with a transverse wall separating them (Fig. 5).

Each dyad megaspore divided further and then gave rise to four megaspore tetrads which are linear in form (Fig. 6). At this point, the integument is 5-6 cells thick and it almost surrounds the nucellus. A micropyle is formed at the point where the integument meets.

The three megaspores at the micropylar end were observed to be degenerating as seen by the decrease in size and disappearance of their nuclei (Fig. 6). The functional megaspore towards the chalazal end enlarged and underwent three successive mitotic divisions and eventually gave rise to the eight nucleated female gametophyte (Fig. 10). This mature female gametophyte derived from a single megaspore is monosporic and is of the Polygonum type.

The female gametophyte is barrel-shaped with four nuclei on the micropylar end and another four at the chalazal end. Three of the nuclei on the micropylar end differentiated into an egg apparatus which consists of the egg cell and two synergid cells. The egg cell had a large vacuole and a nucleus at the periphery. The two synergid cells were hooked with filliform apparatus which appeared as finger-like projections (Fig. 10). The fourth nucleus at the micropylar end moved towards the central part of the cell. Three of the four nuclei on the antipodal end differentiated into antipodal cells, and one of these nuclei migrated towards the central part of the cell to form the two polar nuclei together with the fourth nucleus from the micropylar end.

DISCUSSION

The ovule is tenuinucellate, unitegmic and ana-campylotropous. In the tenuinucellate ovule, no parietal cells developed. All species studied in the family Pittosporaceae are reported to be of this form, and this is the most common type in the angiosperms. The single integument (unitegmic) observed in *P. resiniferum* is a common feature in the family Pittosporaceae. It is also reported to be constant at the family level and is widespread in higher forms of the angiosperms.

The form of the ovule reported for the different species in the family Pittosporaceae are varied. Sheela and Narayana (1966) described the ovules as heminatropous. Davis (1966) described the ovule as anatropous, while Mauritzon (1939) reported apotropous form of ovule. In this study, the ovules were observed to be ana-campylotropous, as also reported by Narayana and Sundari (1978) in Bursaria spinosa.

Maheshwari (1950) reports that in angiosperms, 70% of the female gametophytes are of the monosporic Polygonum type. This is further substantiated in this study. At the present state of knowledge, the Polygonum type is the only type of development reported for almost 80% of the families where development has been described. Only 11 families are characterized by a different single type of development. In the 56 families showing two or more patterns of development, the Polygonum type usually predominates; of the 20 different combinations of types which have been reported to occur in any family, the Polygonum

type occurs in 16 and is apparently the predominant type in at least nine of these combinations. Although there is no information about the total number of species involved, the figures suggest rather strongly that the Polygonum type of development is even more prevalent than what Maheshwari (1950) indicated.

SUMMARY AND CONCLUSIONS

P. resiniferum exhibits the morphological and embryological features of the family Pittosporaceae. The megasporangium (ovule) in this study is ana-campylotropous. A large, single archesporial cell functioned directly as the megaspore mother cell that gave rise to a linear megaspore tetrad. The three megaspores at the micropylar end degenerated, while the chalazal megaspore was functional. The functional megaspore gave rise to the eight-nucleated female gametophyte of the monosporic Polygonum type. These characters, which are reported in the family , are also the most common type in the angiosperms.

Embryological features such as unitegmic, tenuinucellate ovules, single-celled female archesporium, Polygonum type of female gametophyte development, are also recorded in the family Escalloniaceae (Saxifragaceae), (Davis, 1966). This indicates that these two families are probably related to one another and that they may have arisen from a probable common ancestry (Narayana and Sundari,1983).

Based on the observed uniformity of the morphological and embryological features as reported in other species of the Pittosporaceae, the information obtained in this study furnishes a clearer understanding on the basic pattern of growth and development of the female gametophyte in Pittosporum. Data from this study can be used in breeding of different Pittosporum species for better fruit oil in terms of quality and quantity. The embryological characters observed can be used as tools in the classification of angiosperms, since features based on sporogenesis, and gametogenesis are particularly rich in taxonomic characters.

ACKNOWLEDGMENTS

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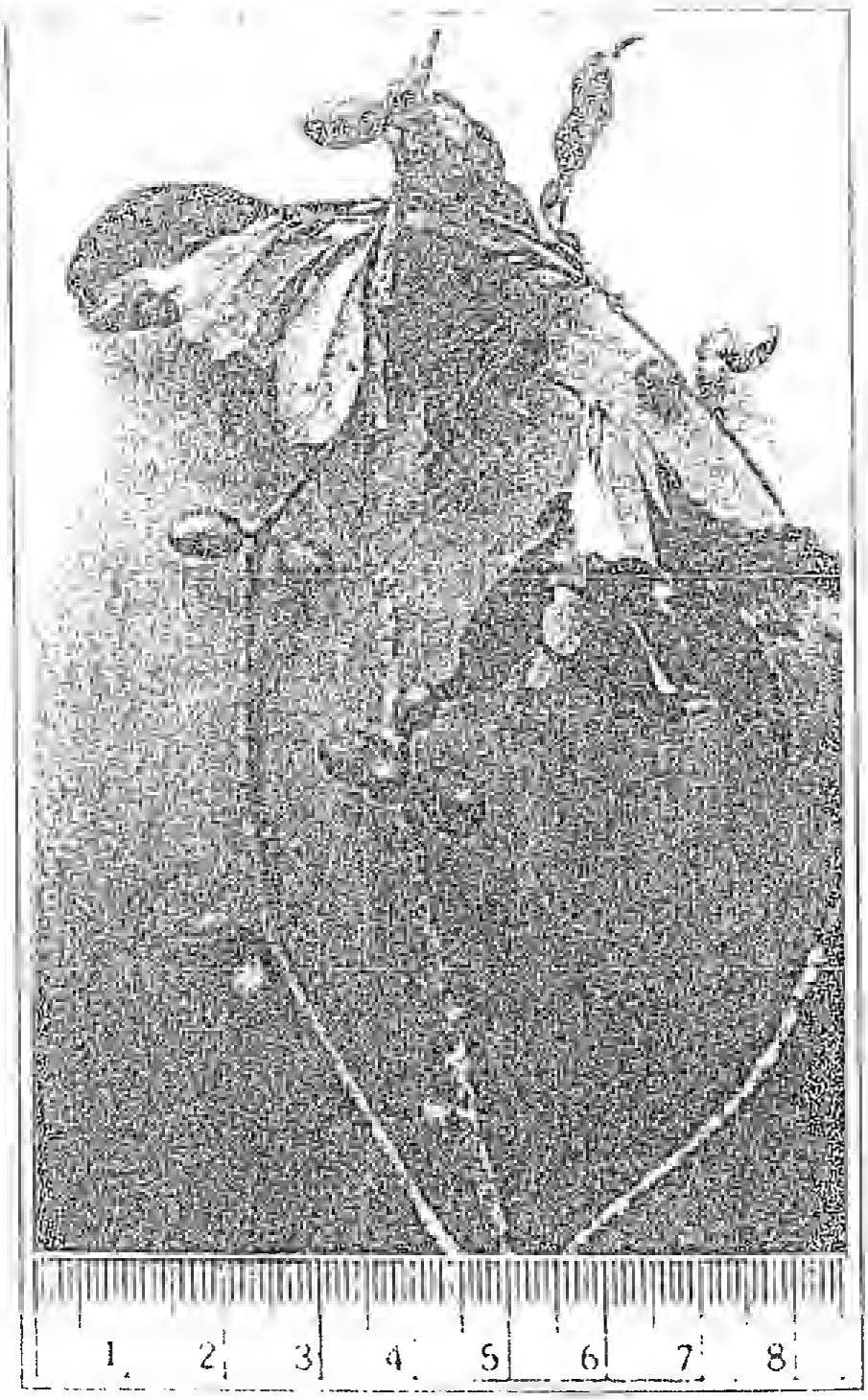

Figure 1. A branch of Pelosporum resinliferum Hemsl. with fruits

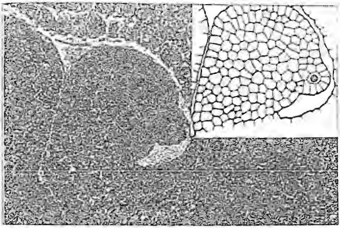

Figure 7. A cross section of a large single megaspore mother cell with a dense cytoplasm and a prominent nucleus. (Bar = 5 µm)

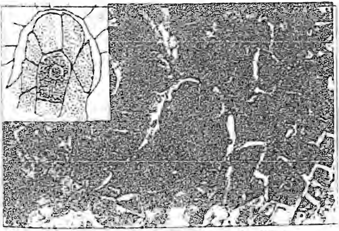

Figure 8. A [illegible] megaspore [illegible]

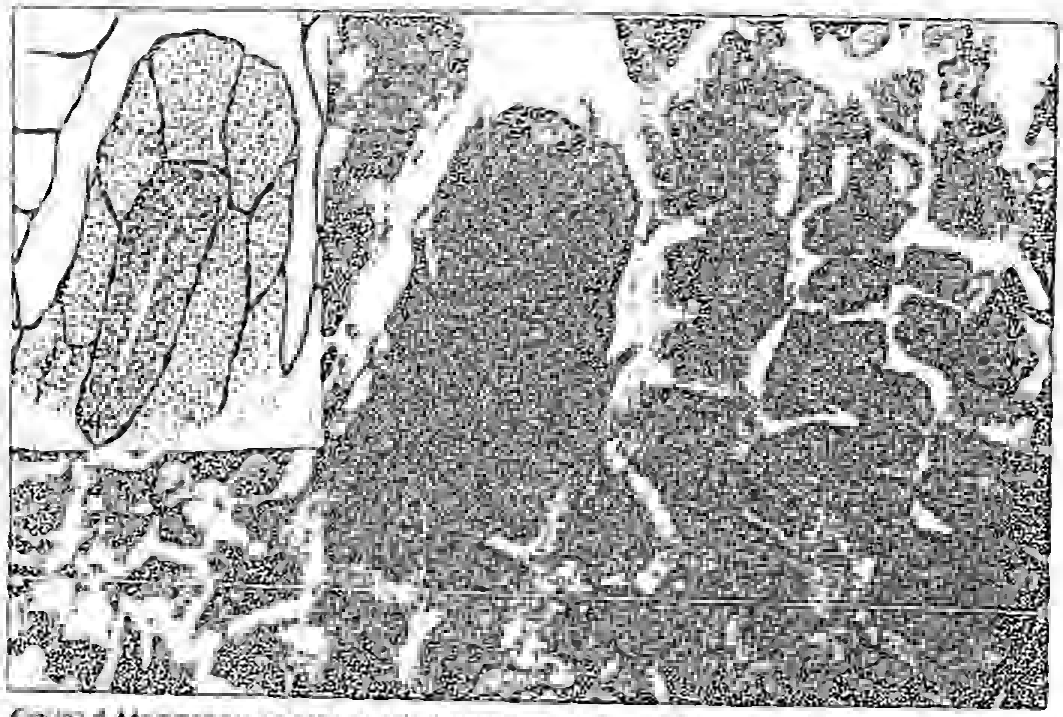

Figure 4. Megaspore mother cell elongating shortly before dividing. Bar = 25.2 um

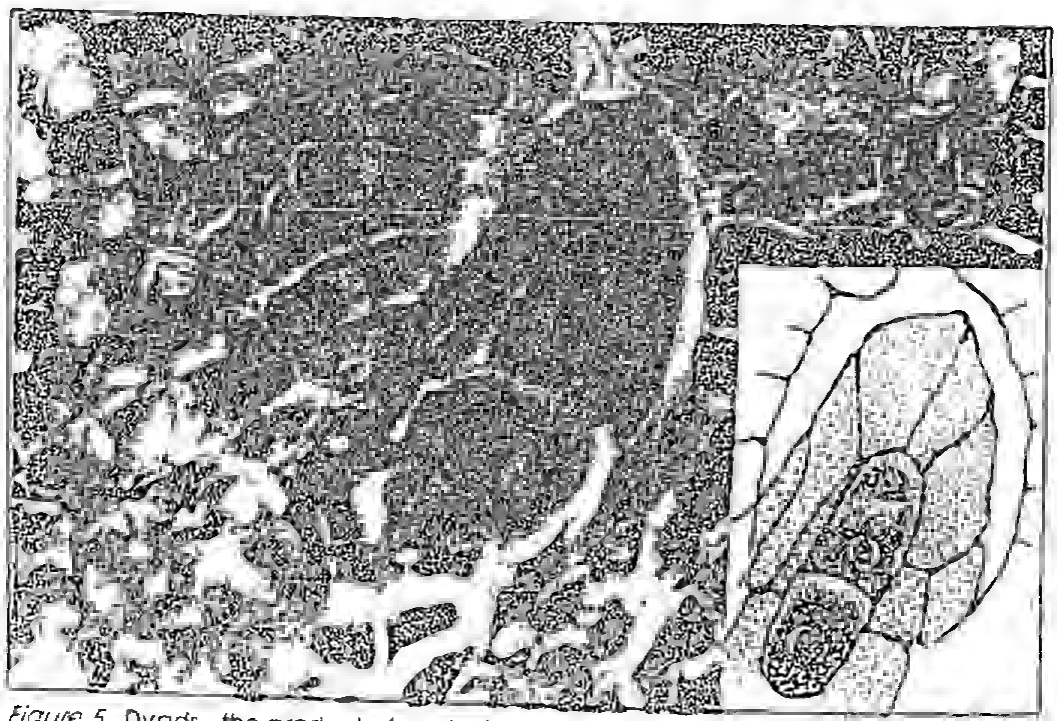

Figure 5. Dyads, the product of meiosis I. Bar = 25.2 um.

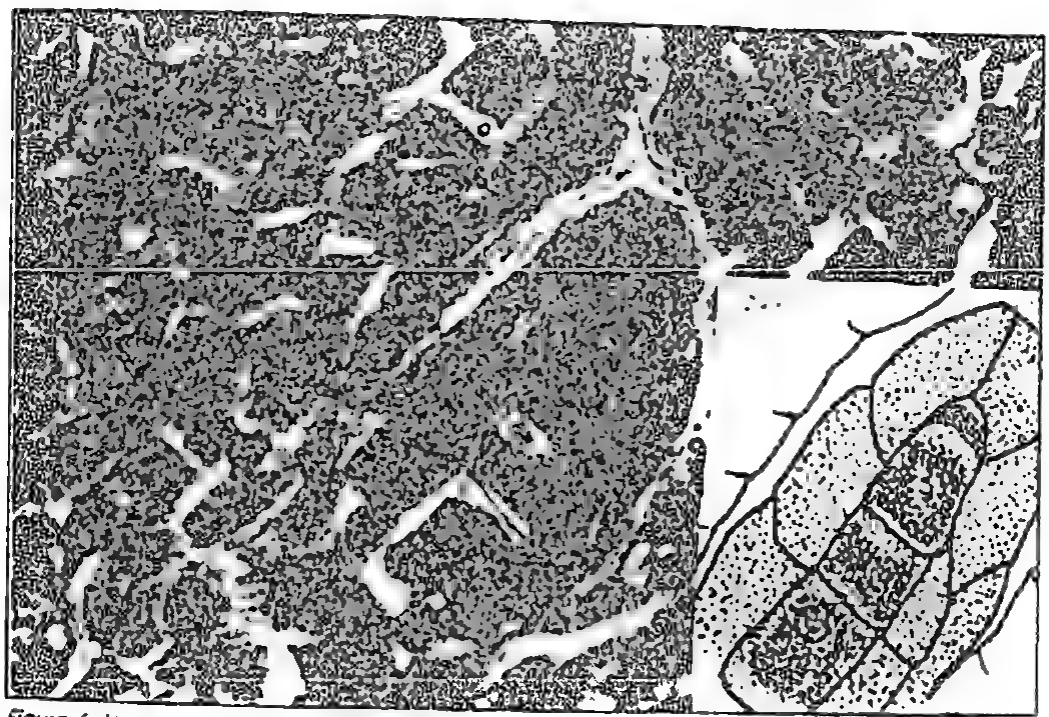

Figure 6. Linear megaspore tetrads with a small cell at the micropylar end about to degenerate. Bar = 25.2 um.

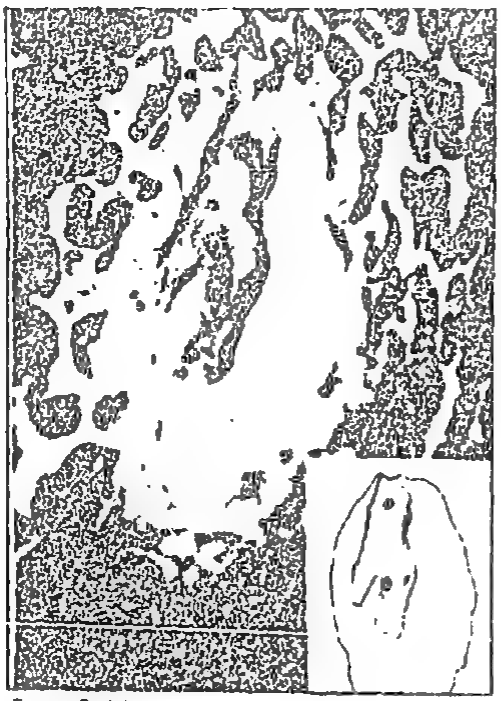

Figure 7. A binucleate female gametophyte. Bar = 28.8 um

Figure 8. A 4-nucleated female gametophyte. Bar = 36um.

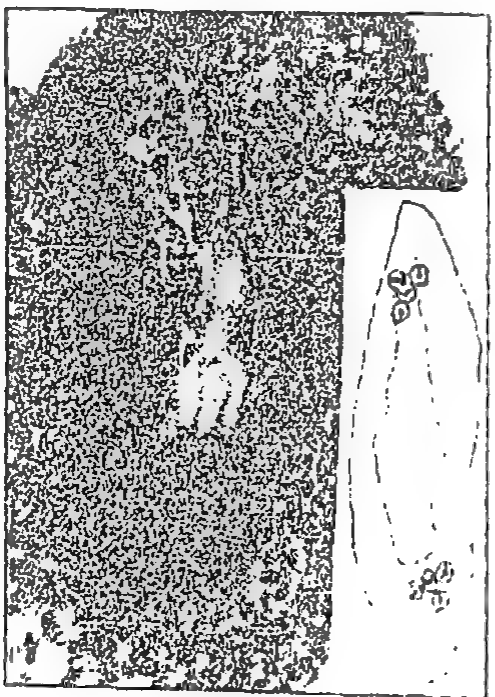

Figure 9. An eight-nucleated female gametophyte with a large vacuole at the center. Bar = 21.6 um

Figure 10. A mature, organized female gametophyte. E =egg; P=polar nuclei Bar = 28.8 um

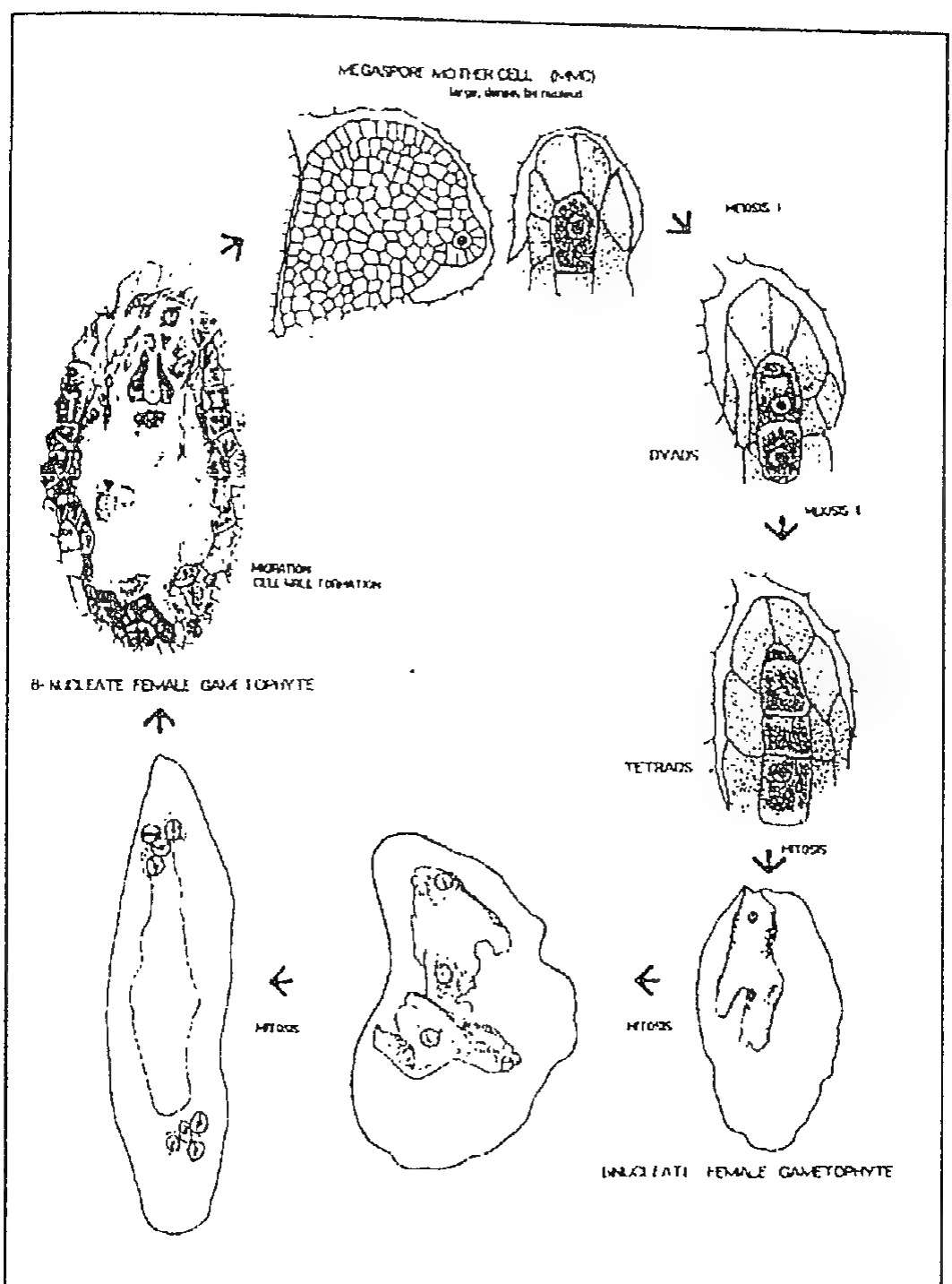

Figure 11. A summary of the development of the female gametophyte of P. resiniferum.

ANTI-INFLAMMATORY AND ANTITUMOR ACTIVITIES OF SEED EXTRACTS OF MALUNGGAY, MORINGA OLEIFERA L. (MORINGACEAE)

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ABSTRACT

The seeds of malunggay, Moringa oleifera, were extracted with distilled ethanol and concentrated under reduced pressure at 40°C. The resulting extract was partitioned between hexane, ethylacetate, butanol and water. The solvent fractions were likewise concentrated under reduced pressure.

The crude ethanol extract of dried seeds inhibited the carrageenan-induced inflammation in the hind paw of mice by 85 % at a dosage of 3 mg/g body weight while the mature green seeds by 77%. The hexane fraction of the crude ethanol extract of the dried seeds also inhibited inflammation by 77 % at the same dosage while both butanol and water fractions inhibited inflammation by only 34%. These results indicate the strong anti-inflammatory activities of the crude ethanol extract and the hexane fraction.

On the other hand, the ethylacetate fraction caused a 267% increase in inflammation and exhibited toxicity. The mice died after oral administration of the fraction. The crude ethanol extract also inhibited the formation of Epstein-Barr virus-early antigen (EBV-EA) induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). At a dosage of 100 (µg/ml the extract inhibited EBV-EA formation by 100% suggesting its antitumor-promoting activity.

INTRODUCTION

The seeds from dried pods of *Moringa oleifera*, commonly known as horseradish and locally known as malunggay, is now becoming another popular "cure-all medicine" not only in the rural areas but also in Metro Manila. It is taken orally at a dosage of 2-4 decoated seeds per day, as cure for arthritis, rheumatism, diabetes, high blood pressure, and heart ailments, among others. The users claim that the mature green seeds are not as effective as the seeds from pods that dry up in the trees.

The medicinal value of the different parts of the plant has long been recognized in folk medicine. An article reviewed the medicinal properties of all parts of the plant as well as the known chemical composition of the seeds with emphasis on the bioactive metabolites. The flowers are taken as diuretic and to cure lingering cough following influenza(Villaseñor, 1994). The pods are taken for their antihelminthic properties (Quisumbing, 1978) while the bark is a cure for asthma (Gutierrez, 1980) The seeds when roasted and powdered are ap-

plied to affected areas for treatment of rheumatism and gout (Phil. Natl Formulary, 1982). The seed oil is used for skin diseases. The seeds have also been found to possess antimicrobial activity (Villaseñor, 1994) As a poultice, the leaves reduce glandular swelling (Quisumbing, 1978) and expel intestinal worms (Gutierrez, 1980). They are also known to be hypotensive and antidiarrheal. The powdered unroasted seeds have been used in many rural areas in Asia and Africa to purify drinking water because of its strong coagulating properties (Villaseñor, 1994).

Because of these many traditional uses of malunggay, several investigations have been conducted to isolate active compounds. Nitrile, mustard oil glycosides and thiocarbamate glycosides have been isolated and found to be responsible for the hypotensive principles of the leaves (Faizi, et al., 1994, 1995). From the extracts of the roots have been isolated a thiocyanate and its glycoside and found to be responsible for the antimicrobial activity of the roots (Eilert, et al., 1981) and seeds (Dayrit, et al., 1990). A mutagen, a rhamnosyl-acetonitrile was isolated from the roasted seeds (Villaseñor, 1988).

This study reports the anti-inflammatory activity of the extracts, providing scientific basis for its use against rheumatism, arthritis and gout. This is the first time that the antitumor-promoting activity of the dried seeds is reported.

MATERIALS AND METHODS

Materials

Chemicals

Technical grade ethanol, hexane, ethylacetate, and butanol were purchased from Air Commercial, Quezon City, Philippines. Except butanol, all the solvents were distilled prior to use. Analytical grade carbon tetrachloride was obtained from Malinkrodt Baker, Inc., carboxymethylcellulose and carrageenan from Sigma Chemical Company, and indomethacin from Merck, Sharp and Dohme.

Plant Material

Sacks of dried seeds of malunggay were collected from Bataan and Naga. Some mature green pods were also collected.

Test Animals

Swiss Webster mice, weighing about 20 g each, were obtained from the University of the Philippines, Los Baños.

Methods

Extraction and Solvent Fractionation

Malunggay seeds were decoated, ground and soaked in distilled ethanol for a few days with occasional stirring. The extract was filtered with filter paper and the filtrate concentrated under reduced pressure at 40°C using a rotary evaporator. The resulting crude ethanol extract of the dried seeds was later partitioned successively between solvents of different polarities like hexane, ethylacetate, butanol and water. The solvent fractions were also concentrated under reduced pressure at 40°C (Figure 1).

Bioassay for Anti-inflammatory Activity: Carrageenan-induced edema method

The anti-inflammatory activities of the extracts were tested by the carrageenan-induced edema method using Swiss Webster mice as test animals. Five mice per test group were used.

The mice, weighing about 20 g each, were starved for 16 h prior to the experiment proper. After fasting, the initial volume of the right hind paw of the mice were measured using a fabricated plethysmometer (Buttle, et al.,1957). The test extracts, dissolved in 2% carboxymethylcellulose (CMC), were administered orally by a feeding gavage at a dosage of 3 mg/g body weight. After one h, 0.04 mL of a 2% carrageenan-saline solution was injected intradermally at the right hind paw of the mice. The volumes of the right hand paw of the mice were again measured 3 h after injection. The % inhibition of inflammation of the test group were compared with the control group which was treated with 2% CMC and carrageenan. Indomethacin, a well known anti-inflammagen, was used as a positive control, given orally by gavage, at a dosage of 0.01 mg/g body weight.

Bioassay for Antitumor-Promoting Activity: the Epstein-Barr Virus - Early Antigen (EBV-EA) test

The effects of the isolates on EBV-EA formation was assayed using Raji cells (virus non-producer), the EBV genome-carrying human lymphoblastoid cells which were cultivated in 10% FBS RPMI 1640 medium (Nissui). The indicator cells (Raji) (1 x 10^6/mL) were incubated at 37°C for 48 h in 1 mL of the medium containing n-butyric acid (4 mM) as inducer, TPA (32 pmol) solution as promoter in 2µl of DMSO and a known amount of test samples dissolved in 5µL of DMSO. Smears were made from the cell suspension. The activated cells were stained with high titer EBV-EA positive sera from nasopharyngeal carcinoma patients and detected by an indirect immunofluorescence technique. In each assay, at least 500 cells were counted and the tests were repeated twice. The average induction was compared with that of the positive control experiments with n-butyric acid and TPA, in

which EBV-EA induction was ordinarily around 40%. These values were taken as the positive control (100%). The viability of cells were assayed against that of treated cells by the trypan-blue staining method

RESULTS AND DISCUSSION

Extraction of the dried seeds with distilled ethanol gave a yellowish brown resinous extract at 10.6% yield. Solvent fractionation gave a hexane fraction at highest yield of 3.8% (% of original weight of seeds), butanol and water fractions at 1.2% each and an ethylacetate fraction obtained the lowest yield of 0.053% (Figure 1).

The anti-inflammatory activity of the extracts of both the dried seeds and the mature green seeds of *malunggay* were indicated by the appreciable inhibition of the carrageenan-induced inflammation on the hind paw of the test mice (Table 1 and Figure 2). At a dosage of 3 mg per gram body weight, the crude ethanol extract of the dried seeds inhibited inflammation by 85% while the extract of the mature seeds gave a 77% inhibition. This difference may partly explain the claim that the dried seeds are more effective than the green seeds.

The hexane fraction of the crude ethanol extract of the dried seeds also exhibited anti-inflammatory activity. At the same dosage of 3 mg/g body weight, the hexane fraction inhibited inflammation by 78%. The butanol and water fractions each gave a relatively low inhibition of 34% and 26%, respectively. The greater activity of the hexane fraction over the butanol and water fractions is significant because the hexane fraction was also obtained in higher yield during solvent fractionation.

On the other hand, the ethylacetate fraction did not inhibit inflammation. Instead it caused about 270% inflammation compared to the control. It was also observed in two separate experiments that 3 out of 5 mice died a few minutes after oral administration of the fraction. These observations suggest that the ethylacetate fraction is not only inflammatory but toxic as well. This toxicity was not observed in the crude ethanol extract probably because of low concentration of the toxic material as evidenced by the low yield of the ethylacetate fraction. When the toxic components became concentrated in the ethylacetate fraction, their toxicity was manifested.

The antitumor promoting activity of the crude extract of the dried seeds was indicated by the appreciable inhibition of the formation of the Epstein-Barr virus-early antigen (Table 2 and Fig. 3). At a dosage of 100µg/mL, the extract inhibited EBV-EA formation by 73 %. Diluting the solution tenfold gave a solution which inhibited EBV-EA formation by 41 % . Further tenfold dilution reduced its effectivity. At a dosage of 1µg/mL, inhibition was only 12%. Inhibition was no longer observed at a very low concentration of 0.1µg/mL.

CONCLUSION

The crude ethanol extract of both the dried and mature green seeds of malunggay possesses strong anti-inflammatory activity when tested on mice with the carrageenan-induced edema method, with the dried seeds more active than the mature green seeds. Solvent fractionation resulted to a hexane fraction which also exhibited strong anti-inflammatory activity and an ethylacetate fraction that caused much inflammation and toxicity.

The crude ethanol extract also possesses moderate antitumor promoting activity when tested using the *in vitro* EBV-EA test.

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Table 1. Treatment Effects of Malunggay Seeds on Carageenan-Induced Inflammation.

Test Samples	Dosage mg/g body weight	% inhibition of inflammation
indomethacin	0.01	69.0
crude EtOH dried	3	85.1
crude EtOH green	3	77.1
hexane fraction	3	78.1
ethylacetate fraction	3	-267
butanol fraction	3	33.9
water fraction	3	25.6

Table 2. Treatment Effects of Extracts of Malunggay Seeds on Epstein-Bar Virus-Early Antigen (EB-VEA) Formation

concentration µg/ml	% inhibition (% cell viability)
100	72.9 (60)
10	40.4
1	11.9
0.1	0

Characteristics of TLC pure compounds

	Developing Solvent	R_f	Color with Vanillin
A	50% Ethyl acatate-hexane	0.33	red
B	50% Ethyl acetate-hexane	0.34	orange
C	75% Ethyl acetate-hexane	0.40	violet
D	75% Ethyl acetate-hexane	0.18	yellow
E	5% Methanol-ethyl acetate	0.48	violet

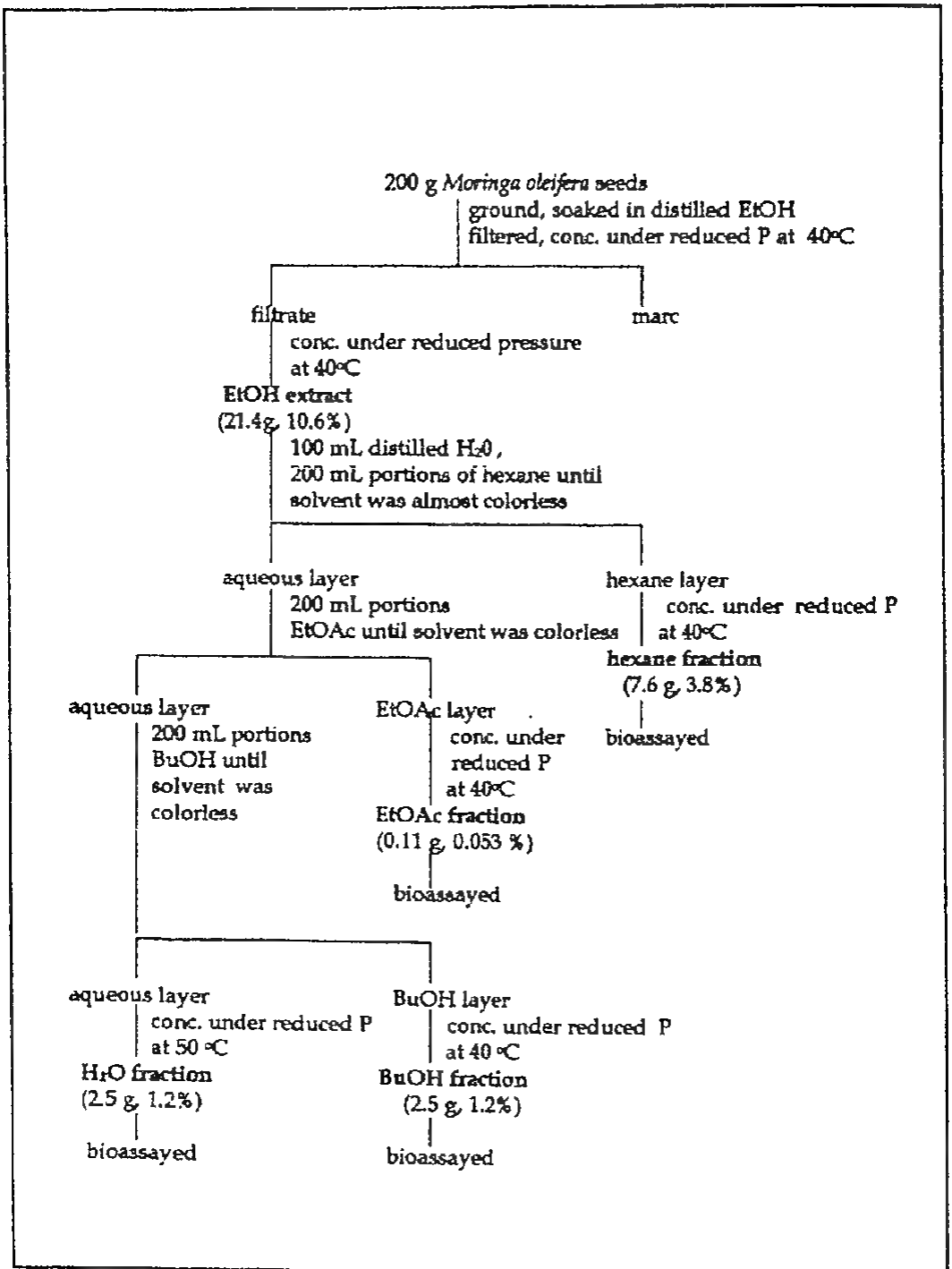

Figure 1. Extraction and Fractionation Scheme.

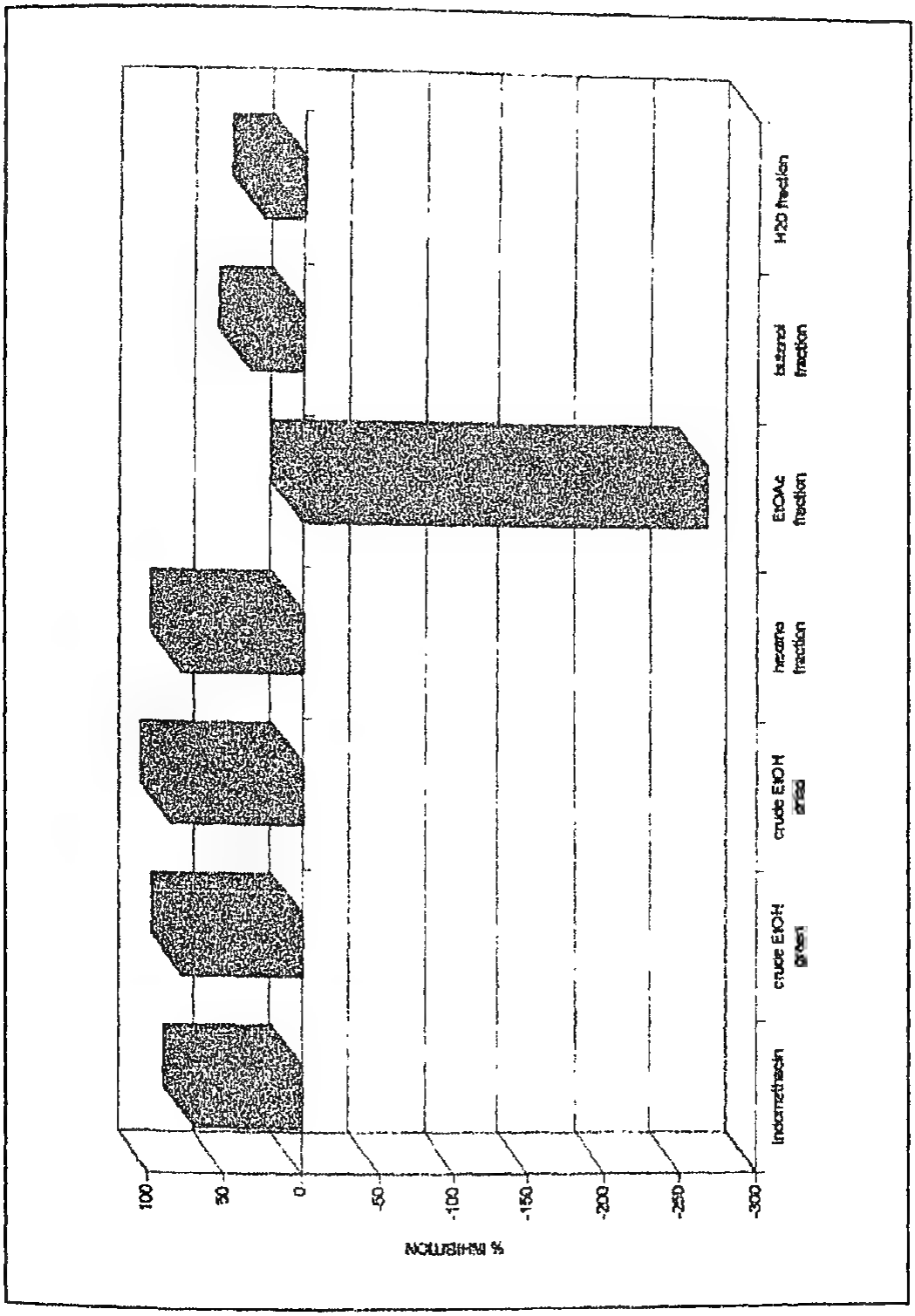

Figure 2. Teatment Effects of Extracts of Moringa oleifera on Carrageenan-induced Inflammation.

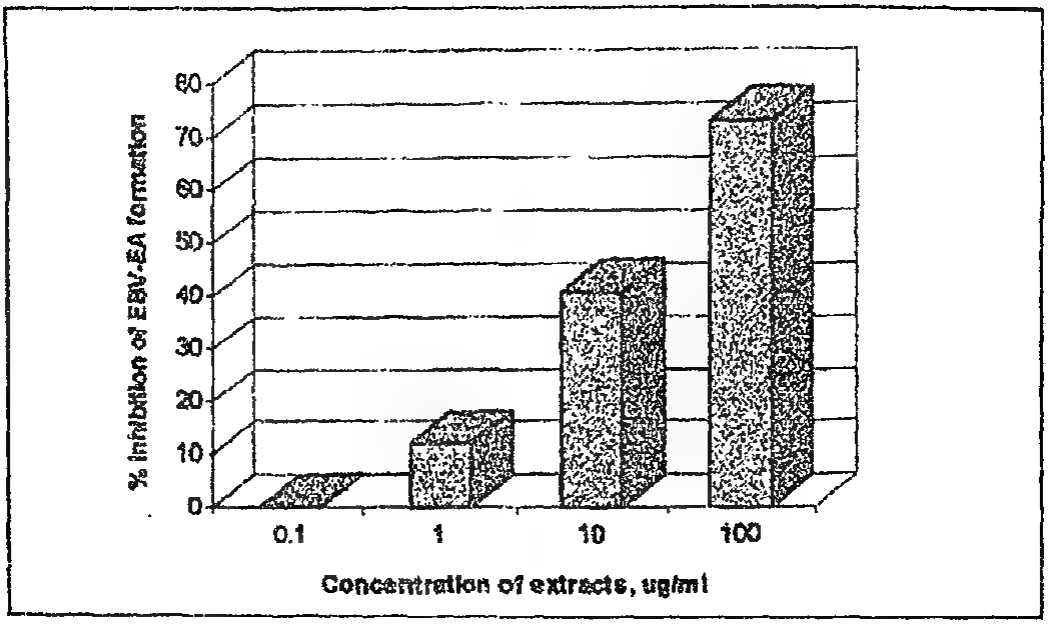

Figure 3. Treatment Effects of Extracts of Malunggay Seeds on EBV-EAFormation.

POLYMERIC ENCAPSULATION OF HEAVY METAL BEARING SLUDGE

BARBARA D.J. TIO[1] and FRANCISCO A. ARELLANO[2]

ABSTRACT

The study attempted to use polymeric encapsulation as a viable alternative for the handling of heavy metal bearing sludges produced from the wastewater treatment operations of semiconductor firm. Two encapsulation materials were used in the experiments; a thermoplastic and a thermoset. Virgin polypropylene resin was used for the thermoplastic encapsulation and the unsaturated polyester resin was used in the thermoset encapsulation.

For thermoplastic encapsulation, a 30 per cent concentration was found to be an ideal value during blending/compounding. The thermoplastically encapsulated experimental slabs were subjected to tensile, impact, morphological, and leaching tests. Results of these tests showed that tensile strength of the materials is decreased as the sludge content is increased while that of impact test almost remain unchanged. Morphological analyses revealed that binding of sludge particles was merely physical entrapment through encapsulation. For the thermoset encapsulation, mixture with 60 per cent sludge gave satisfactory results based on its consistency, moldability and smooth appearance of the encapsulated artwares. The artwares were stored at varying conditions for one month and was found to have no remarkable change. Leaching test confirmed that polymeric encapsulation immobilized heavy metal content of the sludge.

INTRODUCTION

Sludge is normally an unwanted by-product in wastewater treatment. Generally, as higher treatment levels are employed for a particular type of wastewater, more sludge is generated. For instance, the removal of heavy metals from wastewater requires more lime or caustic to remove as much heavy metals as possible during precipitation process. This eventually results to increased volume of sludge.

The physical and chemical characteristics of sludge technically and economically dictate the effective means ofdisposal. Ultimate disposal usually employed are landfilling and incineration. Disposal of toxic heavy metal bearing sludges poses a major problem because of possible secondary pollution. If these sludges are incinerated, some constituents become air-borne pollutants; if washed out in scrubbers, the constituents become water

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borne. One of the practical disposal methods is landfilling. However, without the proper handling and pre-disposal treatment processes, groundwater contamination due to leaching out of these toxic substances presents an imminent danger. It is, therefore, imperative that emphasis be given to the re-use and recovery of waste so that these materials may be utilized as raw material input for a particular process instead of just being discarded. If recovery of these waste materials is not feasible, these must be treated to reduce their toxicity level by rendering the toxic constituents immobile or non-hazardous.

The sludge from the semiconductor industry is one of the identified wastes which cannot be disposed of directly in landfills without undergoing solidification/stabilization process as recommended by the US Environmental Protection Agency (USEPA) (Annex A). In view of this, solidification/stabilization technology has been identified to be appropriate for this particular sludge. Although solidification and stabilization are mechanically the same, they have distinct purposes. Solidification is a traditional process aimed at solidifying wastes by reacting its free water with a binder like cement or lime. On the other hand, stabilization is an emerging technology which purposely treat hazardous waste to a minimum leachability standard as determined by the Toxicity Characteristic Leaching Procedure (TCLP). This is done by reacting hazardous waste with a binder to fix or encapsulate the hazardous constituents in the solid matrix. Majority of the commercial stabilization processes (Annex B) are performed with inorganic binders. There are various organic binders that have been proposed but their performance have not been fully studied to date.

This study dealt on the stabilization by polymeric encapsulation of heavy metal sludge generated by a semiconductor manufacturing company. The polymeric encapsulation is an emerging technology and has technical merits which include; improving the handling and physical characterisitics of the waste and reducing or minimizing pollutant solubility. Since there is no current widespread usage of this technology, the study has been purposely aimed at generating baseline data which may have considerable future applications.

MATERIALS AND METHODS

The encapsulation method is currently done in other countries, especially those with strict enforcement of environmental regulations, for the management of hazardous waste particularly toxic heavy metal laden sludges.

Materials

The study was conducted at the Polymers Section of the Materials Science Division (MSD) of the Industrial Technology Development Institute - Department of Science

and Technology (ITDI-DOST) in Bicutan, Taguig, Metro Manila. The chemicals used were of analytical reagent grade. The bulk of materials used were sludge, polypropylene, and unsaturated polyester resin.

Sludge

The sludge used in the study was provided by a semiconductor firm based in Sucat, Parañaque. It was obtained by alkali precipitation of wastewater in the plant. It is packed in black plastic bags placed inside tin cans. The filter-pressed cake sludge has a gray to greenish color with no objectionable odor.

Polypropylene

Injection-grade cosmoplene polypropylene (W 101) with a melt low of 5 grams per 10 minutes was used in thermoplastic encapsulation. Polypropylene is the lightest of the common plastics, with a specific gravity of 0.90. General physical and electrical properties of polypropylene is similar to those of high-density polyethylene. This material was imported from Singapore.

Unsaturated polyester resin

For the thermosetting encapsulation, the unsaturated polyester resin (R 10-103) casting grade and methyl ethyl ketone peroxide were used as resin and catalyst respectively. These materials were locally available.

Methodology

The study was conducted following the experimental design presented in Figure 1. The polymeric encapsulation, however, was limited to the type of sludge supplied by a particular semiconductor company. The possibility of using commercially available coupling agent or anti-oxidant for the improvement of the dispersion of sludge in the plastic matrix was not tried.

Sample Preparation

The wet sludge obtained from the semiconductor plant was dried, milled and screened prior to subsequent characterization and encapsulation. Drying, milling and screening were done to transform the wet sludge into powdered form which was more manageable. Moisture content of the sludge was determined during drying.

Drying of Sludge

The drying of sludge samples was undertaken to standardize mixing ratio of sludge and polymer materials. It was also done to assess the sludge dewatering process employed

in the semiconductor plant where the sludge was generated. The wet sludge, approximately 20 kg., was spread on enamel trays and placed inside the drying oven for 24 hours at a constant temperature of 100°C ±5°C.

Moisture Content Determination

The moisture content determination of the sludge was done by Drying Oven Method. Five replicate samples of sludge with varying weights were spread on trays and placed inside the drying oven following the conditions done for moisture content determination. The samples were allowed to cool to room temperature in a large dessicator. The difference between the original sample weight and the weight after drying was used to determine the moisture content of the filter-pressed sludge.

Grinding of Dried Sludge

The dried sludge, approximately 2 kg., was placed inside a 5 kg. capacity jar mill with 2 kg. grinding balls. Ballmilling was done batchwise at 60 revolutions per minute (rpm) for different periods of 1, 3 and 5 days.

Screening

The milled sludge was placed in a vibrating screen. Particles passing 100 mesh were set aside for characterization and encapsulation. The oversized particles were put back in the jar mill for regrinding.

Sludge Characterization

Characterization of sludge was undertaken to provide basic information on its treatability and allow some estimate to be made on treatment processes and operating parameters. Physical testing of sludge also helped to demonstrate the relative success or failure of stabilization and solidification techniques.

Hardness

To determine whether the sludge contain abrasive materials or whether it is resistant to abrasion, the hardness test was carried out.

The hardness of dried sludge samples was obtained by scratching the dried sludge samples with the reference minerals/materials in the Mohs' scale. Three samples of dried sludge in pebble form were used in the determination.

X-Ray Diffraction Analysis

X-ray diffraction (XRD) is an instrumental technique for identification of crystalline materials. This method was used in the characterization of sludge in order to know if minerals or crystalline materials were present in the sample which might interfere in plastic processing and affect properties of encapsulated sludge.

XRD analysis of the dried sludge was obtained using Shimadzu X-ray Diffractometer (VDR-2) set at 30 KV and 20 mA to give CuK- radiation. The slit system was set at 2 while the goniometer was adjusted to operate at 2 per minute with a time constant at 2000 counts per second and the recorder chart speed at 20 mm per minute.

Elemental Analysis

To quantitatively and qualitatively determine the heavy metals present in the sludge samples the elemental analysis was undertaken.

The heavy metals in the sludge were determined using Scanning Electron Microscope (Model JSM-T330A) with energy dispersive X-Ray Spectrophotometer (EDS) and with microprocessing unit. Representative micro area of the sample was scanned by electron beam operating at 15 kV accelerating voltage and 100x magnification.

Differential Thermal Analysis

Plastic processings, especially during blending/compounding, are usually done within specified temperature limits, from 100 to 200°C,and the incorporation of inert matter like sludge into the plastic may have adverse thermal reaction during compounding. Therefore, it is important to know the physical property of sludge (and/or its reaction products) as a function of temperature. The thermal analysis of the sludge was obtained using a Differential Thermal Analyzer (Model Rigaku Thermoflex TG 8110). The equipment was set at a heating rate of 10°C/minute, chart speed of 2.5 mm/minute, with maximum working temperature of 1000°C in an air atmosphere.

Thermoplastic Encapsulation

The thermoplastic encapsulation refers to stabilization of toxic material using thermoplastic material like polypropylene as organic binder. At present, encapsulation of this kind is used to a limited extent with radioactive waste. The concept of thermoplastic encapsulation is similar to the addition of filler into the plastic matrix. The powdered sample with particle size of at least 10 microns were used in the thermoplastic encapsulation.

Blending/Compounding of Sludge and Polypropylene

The powdered sludge was mixed with polypropylene at a sludge : polypropylene ratios of 4:36, 8:32 and 12:28. Blending of sludge and polypropylene at a ratio higher than 12:28 mixture was discontinued due to the difficulty encountered in achieving an intimate mix of sludge and polypropylene. The total weight of sludge and propylene mixture per blending was 40 grams. Mixing time of 5, 10 and 15 minutes were tried.

The blending/compounding of sludge and polypropylene was done using a Brabender Plasticorder with microprocessing unit. The equipment was set at 190°C and 200°C and residence time of 3, 4 and 5 minutes.

Compression Molding

The blended sludge and thermoplastic from the Brabender were formed into slab test specimens using a Shinto Compression Molding Machine which was set at operating pressure of 50kg/cm2, temperature of 190°C and 200°C and residence time of 3, 4, and 5 minutes.

The weight of the blended sludge and thermoplastic was approximately 90 grams per molding. The mold used has a dimension of 10" x 8" x 0.5". The sample was spread over the molding trays and was automatically compressed by the machine.

Product Testing

The plastic slabs produced in the thermoplastic encapsulation were air-dried for two (2) weeks and their physical properties were determined as follows:

Tensile Strength Determination

Tensile strength was determined to evaluate the effect of sludge on the physical properties of plastics. The tensile strength of plastic slabs formed by compression molding was determined following ASTM D638 Standard Method.

Dumbbell-shaped specimens (virgin pp, pp with 10% sludge, pp with 20% sludge and 30%sludge) were prepared by die cutting the slab-formed specimen. These were then conditioned for 40 hours prior to testing. Thickness and width of the different specimens were determined prior to tensile testing. Two light center marks were made on each test length, exactly 2 inches apart. The specimen between these gage marks was the material analyzed under test. The specimen was clamped in a testing machine and load was applied. The testing machine (model UCT-5T) had a cross head and chart speed of 50 mm per minute and 1.3 mm per minute, respectively.

Impact Test (Izod Type)

The determination of the resistance to breakage by flexural shock or impact resistance of plastics was done using ASTM Method D256.

The conditioned specimens (virgin pp, pp with 10% sludge, pp with 20% sludge, and pp with 30% sludge) measuring 2.5 in x 0.5 in. were subjected to notching. A notch was machined into rectangular test specimens. A free- swinging pendulum was allowed to break the specimen. The recorded impact strength is the measure of work done in breaking test samples.

Morphological Analysis

To examine the microscopic arrangement of sludge within the plastic matrix the scanning electro microscope was used. The test specimens (virgin pp, pp with 10%, 20% and 30% sludge) were cut under liquid nitrogen into spherical- shaped samples with diameter of about 4 mm. The use of liquid nitrogen is necessary in order to arrest possible thermal stress on the plastic slabs which will be reflected in the micrographs. The test samples were coated with 200µm gold using the Ion Sputtering equipment for conductivity purposes. Morphological analyses were observed using JEOL-T330 Scanning Electron Microscope set at 200x, 1000x and 2000x magnifications.

Leaching Test

The three samples with sludge concentration of 10%, 20% and 30% from thermoplastic encapsulation were subjected to leaching test. The procedure adapted for leaching test was the modified Toxicity Characteristic Leaching Procedure (TCLP) and American Nuclear Society Leach Test (ANS, 1986). For ease of operation, the leach test done at static state. The leaching solution used was acetic acid with a pH of 3.0 and with liquid to solid ratio of 20:1. To determine the trend of leaching, the solution was analyzed for heavy metal content after 14, 28 and 56 days using Atomic Absorption Flame Emission Spectrophotometer (Model No. AA-680).

Thermoset Encapsulation

Thermoset encapsulation refers to stabilization of heavy metal bearing sludge with the use of thermosets. Thermosetting polymers, numbering less than the thermoplastic group, possess quite different characteristics. They form a rigid, hard and often brittle, infusible mass once they polymerize.

The sludge sample which passed the 60-80 mesh screen was used for thermoset encapsulation.

Casting of Sludge and Unsaturated Polyester Resin

The powdered sludge was used as filler in the production of molded artwares. The formulations tried for resin and sludge resin and sludge mixture were 20:80, 30:70, 40:60, and 50:50.

The resin was slowly added to the sludge inside the plastic beaker and was thoroughly mixed until no lumps appeared on the mixture. About 1.5% by weight of methyl ethyl ketone peroxide was added to the mixture as catalyst. The mixture was poured into rubber moulds and was allowed to harden for at least thirty minutes before releasing from the moulds.

Stability Test

The molded artwares (rabbit and mouse) were cured in a dry cool place for a month. Afterwards, representative samples were placed outdoor and in an air conditioned room for another one month. Both samples were inspected from time to time for any noticeable physical change, like vaporization of water through change in weight, and softening of mixture.

Leaching Test

The molded artware with 60% sludge concentration was subjected to leaching test. Likewise untreated sludge was also subjected to leaching test to comparatively evaluate the performance of polymeric encapsulation. The procedure adapted for leaching test was the same as with that of thermoplastic encapsulated sludge, the modified Toxicity Characteristic Leaching Procedure and American Nuclear Society Leach Test. Acetic acid with pH 3.0 was the leaching medium with the liquid to solid ratio of 20:1. The aliquot portion was analyzed for heavy metal content after 14,28 and 56 days using Atomic Absorption Flame Emission Spectrophotometer (Model No. AA-680).

RESULTS AND DISCUSSIONS

Materials

Instrumentation techniques were adapted in the conduct of the study, therefore, the use of additional chemical reagents was minimized. The bulk of material requirements consisted of sludge , virgin polypropylene resin and unsaturated unsaturated polyester resin (5 liters).

Sludge

The solid cake sludge which was obtained from a semiconductor firm has pasty characteristics, green in color, sticky, with fine texture and homogenous in particle size. The wet sludge cake which was provided by the semiconductor plant, weighed about 30 kg.

Polypropylene

The polypropylene used in the study was virgin resin. An earlier attempt was made to use plastic wastes for the encapsulation but this was discontinued due to non homogenous properties of plastic wastes, like the presence of different colorants/dyes and other plastic additives.

Polypropylene was chosen over virgin polyethylene resins due to its superior properties. Although both polyethylene and polypropylene fall under the category of crystalline polymer, wherein they have more regular molecular arrangements, polypropylene has an outstanding resistance to flex and stress cracking, has excellent chemical resistance and impact strength, good thermal and dimensional stabilities (Baird, 1971).

Unsaturated Polyester Resin

The unsaturated polyester resin used in the study has fluid consistency and with strong aromatic odor. It is produced from the polymerization of certain alcohols and acids. The properties of polyester are varied as to form in which they are processed.

Sample Preparation

The filter-pressed sludge exhibited physical properties similar to clay. Therefore, procedures for drying, moisture content determination, grinding and screening of clay materials were adapted during sludge preparation.

Drying of Sludge

The wet sludge was not subjected to temperatures exceeding 110°C to avoid possible thermal degradation of its composition. Drying time of 12 hours produced a moist soil-like sludge while drying time of 24 hours produced a brittle clinker-type mass.

Moisture Content Determination

The sludge was found to contain moisture ranging from 68-73%. The moisture content arithmetic average based on five (5) replicate samples tested was 71%.

Grinding of Dried Sludge

Ballmilling of dried sludge for one (1) day produced pebbles. Coarse and grainy particles were produced by ball milling for 3 days. Continuous ball milling for 5 days produced powdered and fine particles.

Screening

Almost 80% of milled sludge passed through 100 mesh screen. The oversize materials, about 20%, were put back inside the jar mill for regrinding.

Sludge Characterization

Sludge characterization was important prior to designing encapsulation process that might be adapted in the study. Characterization study included the determination of hardness and heavy metal contents of the sludge. X-ray diffraction and thermal analyses were also done on the sludge sample.

Hardness

The degree of hardness was determined to ascertain whether the sludge will offer resistance to abrasion or if the sludge is abrasive. The abrasiveness of sludge can adversely affect the rotary blades of the equipment which will be used during blending and compounding. Hardness obtained using the Hardness Mohs' scale was grade 2 to 3, a hardness between gypsum and calcite. It could be concluded that the sludge had no abrasive material because as specified in the Mohs' scale, gypsum can be easily scratched by fingernail while calcite can be readily cut by knife (Katz, 1987).

X-ray Diffraction Analysis (XRD)

The presence of crystalline materials in appreciable quantity can damage the equipment used in plastic processing especially the Brabender-Plasticorder which has very sensitive mixer blades. Moreover, dispersion of inorganic filler like sludge in plastic matrix is greatly affected by the presence of minerals. In order to assess the mineral content of the sludge, x-ray diffraction was undertaken. Results of the x-ray diffraction analysis revealed that the sample contains <-quartz silica. This is exemplified by a high intensity peak with d-value of 3.35 A (see Figure 2). The slight increase in baseline at 20° - 40° (2O) suggested that the sample also contains amorphous materials. The presence of relatively small amounts of silica in the sludge is an indication that it could not affect its dispersion in the plastic matrix nor could damage the equipment.

Elemental Analysis

Results of the elemental analysis showed that the major component of the sludge, Copper comprises 88.47 per cent by weight, followed by tin which is 6.86%. Iron, phosphorous, silicon, sulfur and aluminum were also present but at lower concentrations relative to Cu (see Table 1). Since the sludge resulted from precipitation process, it was presumed that most of these heavy metals were in the hydroxide form. Although Copper is not toxic to humans, disposal of sludge containing Cu concentration of about 880,000 ppm, without stabilization or encapsulation process, is repugnant.

Differential Thermal Analysis

Because it is nearly impossible to detect specific types of compounds which might be present in the sludge, the differential thermal analysis was undertaken. The principle of this method is that the heat effect associated with a reaction is related to the rate of reaction. This method also helps in detecting the general behavior of sludge when subjected to high temperatures which is used as the basis in plastic processing.

Figure 3 shows the differential thermal analysis of powdered sludge. The peaks of the curve at 82.7°C and 152.5°C were attributed to the loss of adsorbed water, the exothermic peak at 278°C might be due to decomposition or dissociation reactions of copper hydroxide, copper sulfate and/or phosphoric oxysulfide. The endothermic effect at 838.7°C might be due to the melting of SnS. So far, results of the thermal analysis did not indicate any adverse thermal reactions of sludge which might hamper the processing temperature of polymeric materials.

Thermoplastic Encapsulation

The sludge and polypropylene during the encapsulation do not interact chemically but each sludge particle is captured within the plastic matrix. Thus, heavy metal content of the sludge, copper in this case, was bonded to or surrounded by an impervious covering.

Blending/Compounding of Sludge and Polypropylene

The intimate blending or mixing of sludge and polypropylene resin was undertaken using the Brabender-Plasticorder. The equipment is a precise measuring equipment for testing the processibility of thermoplastics, thermosets and many plastifiable material.

For the blending of polypropylene resin and sludge, the plastogram (torque vs. time) was measured. The measuring principle was based on the fact that the resistance which the test material - the polypropylene and sludge, put up against rotating blades and rotors in the measuring mixer was made visible. At the same time, a stock temperature diagram was recorded.

The plastogram of pure polypropylene resin was shown in Figure 4. The plastograms of polypropylene resins with 10%, 20% and 30% by weight sludge were shown in Figures 5, 6 and 7, respectively. The torque is measured in Newton-meter (Nm), stock temperature is in degrees centigrade (°C) and test time in minutes (min.)

As reflected in the different plastograms, the sludge did not offer much resistance against the rotating blades compared with virgin polypropylene resins. After about one

(1) minute of blending, the measured torques were 15.5, 11.0, 13.9 and 10.9 Nm for pure polypropylene resin, 10%, 20% and 30% sludge, respectively. This could mean that force required in blending thermoplastic is not greatly affected by addition of sludge. In fact it could minimize the force required in mixing samples as reflected by the decreasing values of torque as the amount of sludge was increased.

The softening temperature for polypropylene ranges from 160 to 170°C, therefore the blending/compounding of polypropylene and sludge was set at 190-200°C. Blending at this temperature was likewise considered to counteract the tendency of sludge particles to form neutral agglomerates due to the absence of polar groups that could initiate intimate contact between sludge and nonpolar polymers like polypropylene. However, blending/compounding above 205°C was not done to avoid possible thermal degradation of polypropylene. This process of thermal degradation refers to breaking down of molecular structure or production of low molecular weight fragments which are often flammable and unstable.

The optimized blending time for sludge and polypropylene mixture, based on the four (4) plastograms, falls between 5 and 10 minutes. At this time, the torque becomes quite stable as shown by relatively straight line in the graph of torque vs time. After about 10 minutes of blending/compounding, the corresponding torques for virgin polypropylene resin, 10%, 20% and 30% by weight sludge mixtures are 9.5 Nm, 7.7 Nm, 9.8 Nm, and 8.9 Nm, respectively (Figures 4 - 7). Although the torque value for 20% sludge changed from 10.1 to 9.7 Nm (see points X5 and X6 of Figure 6), it went up to 10.0 Nm after 7 minutes and remained almost constant until 10 minutes blending was reached. Blending time for more than 10 minutes, although technically feasible, is not economically viable due to high energy process requirement.

Compression Molding

Compression molding at temperatures lower than 190°C produced plastic slabs with tiny voids. These tiny voids or white specks might be attributed to the metallic particles like copper, iron and aluminum that are encased in oxide films when exposed to air. Curing time of less than five (5) minutes produced plastic slabs with large voids on the mold sides. Similar result was observed when the newly compressed hot plastics were dipped in water for cooling purposes. This could be attributed to the entrapment of air on exposed sides when there was sudden change in pressure and/or temperature. Based on the smooth appearance of plastic slabs and absence of voids in them, the optimum molding temperature during compression molding was 190°C and curing time of five (5) minutes. To facilitate the release of plastics from the hot molds and eliminate the formation of voids along the sides of molded plastics, the molded plastic was allowed to cool at room temperature for about ten (10) minutes.

Product Testing

The plastic slabs produced by compression molding of blended sludge and polypropylene resin have smooth appearance and brownish-green in color. Tiny specks were visible for plastic slabs containing 30% sludge, none was observed for slabs containing 10% sludge. The plastic slabs after conditioning or air drying for two (2) weeks were cut into desired sizes for testing.

Tensile Strength Determination

Sludge as filler affects tensile strengths of molded plastics according to packing characteristics, sizes, shapes and bonding (Katz, 1987). Tensile strength is one of the most important properties of a material. It is the force necessary to pull the specimen apart and kind of stretching that occurs during breaking.

The virgin polypropylene resin gave the highest average values of stress and strain, 4123.04 psi and 0.02557 in./in., an indication that it is the toughest. The average tensile stress values in psi of thermoplastics with 10%, 20% and 30% by weight sludge are 3306.58, 2868.51 and 2604.11 *(Table 2)*, respectively. The tensile strength decreases as the sludge increases. The strain value has also a decreasing trend as the sludge concentration is increased. The average strain value for virgin polypropylene resin is 0.2557 in/in., while average strain values for 10%, 20% and 30% by weight sludge are 0.05164, 0.03074 and 0.02826, respectively *(Table 2)*. Based on the strain values obtained, virgin polypropylene resin is the most elastic among the four samples *(Figure 8)*. This is attributed to the presence of crystallite regions in virgin sample which is affected by the presence of sludge.

The plastics with 30% sludge by weight had the highest average value for modulus of elasticity, 99,546.302 psi. Modulus of elasticity is the stress necessary to deform the material confirmed that polymeric encapsulation of heavy metals bearing sludge produced hard plastics which was charaterized by high modulus of elasticity. The plastic becomes harder as the sludge concentration in the thermoplastic encapsulation increases (Table 2). Modulus of elasticity is the stress necessary to strain or deform the material elastically to twice its original length. Most rigid materials have high modulus of elasticity values since a large stress will be required by only a small strain. Of the four samples, plastic with 30% sludge is the most rigid or the hardest. The plastic becomes harder as the sludge concentration in the thermoplastic encapsulation is increased as reflected in the increasing trend of modulus of elasticity, 16,667.21 psi for virgin polypropylene; 65,094.06 psi, 94,699.414 psi, and 99,546.302 psi for plastics with 10%, 20% and 30% sludge concentration by weight. Polypropylene being crystalline was tough because the presence of crystallite regions favorably increased the tensile stress and elongation or strain *(Figure 8)*. The virgin polypropylene was more elastic than those polypropylene with sludges. The elongation of pure pp reached up to as much as ten times of its length as shown in the stress-strain curve.

Impact Test

Impact strength is of great practical importance and sometimes simulate actual conditions of use. Results of impact tests shown in Tables 3 and 4 gave average izod impact strengths (in ft-lbf/mm) of 0.00408, 0.00404, 0.00404 and 0.00407 for virgin polypropylene and thermoplastics with 10%, 20% and 30% sludge by weight, respectively. The highest impact strength recorded was 0.00467 ft-lbf/mm from thermoplastic with 30% sludge, while 0.00386 ft-lbf/mm was the lowest impact strength reading from thermoplastic with 10% sludge *(Table 3)*.

Based on the values obtained for the izod impact strength, it is evident that the impact strengths of the encapsulated thermoplastics were not adversely affected by the incorporation of heavy metal bearing sludge. In fact, high sludge concentration somehow contributed in improving the cohesive strength of the filled plastics.

Morphological Analysis

Comparative electron micrographs of thermoplastic without sludge and with 10%, 20% and 30% sludge concentration by weight at 200X, 1000X and 2000X magnifications are shown in Figure 9, 10, 11 and 12, respectively. Micrographs of virgin polypropylene resin *(Figure 9)* have well defined contours and relatively dense. Micrographs of thermoplastics with 10% sludge *(Figure 10)* are marred with tiny specks which are more visible in the micrographs of thermoplastics with 20% and 30% sludge *(Figure 11 and 12)*. The 2000x magnifications revealed the presence of voids which are more prevalent in samples with more than 10% sludge. These voids which were made visible by micrography might be possibly caused by air entrained around the heavy metals. The morphological analysis showed that the particles of sludge adhere or glued" unto the plastic matrix as reflected by the presence of tiny specks in the micrographs. This clearly indicates that encapsulation of heavy metal bearing sludge with thermoplastic is merely a physical combination. There is no marked chemical bonding or reaction that has undertaken between sludge and polymer.

Leaching Test

The acidic solution used for determining leachability of polymeric encapsulated sludge was subjected to heavy metal analysis. Since Copper was the major constituent of the sludge sample, the solution was analyzed for its Cu content for ease of operation. Results of the analysis is shown in Table 8.

The copper leached in mg/l, from thermoplastic encapsulated sludge were 1.56, 1.96 and 1.25 for 10%, 20% and 30% sludge, respectively. After 28 days, the plastic with 30% sludge gave the highest copper leached followed by sample with 20% sludge. The 10% sludge had the least copper leached, 1.83 mg/L. The same pattern of copper leached

was observed after 56 days.

The leaching test done on the polymeric encapsulated waste was a simple process which probably suited to describe the short-term (well-managed site with waste form intact) leaching behavior of the encapsulated waste, however, the results obtained here cannot be used in extrapolating the long-term (waste that has been subjected to many years of environmental stress) leaching characteristic of the waste.

Thermoset Encapsulation

The sludge samples used in thermoset encapsulation should have uniform particle diameter of not larger than microns. The particle diameter greatly influenced the packing density and texture of the molded sludge and thermoset.

Casting of Sludge and Unsaturated Polyester

Molds that were used for the casting of sludge and unsaturated polyester resin are made up of silicon rubbber. The pouring of the mixture (sludge and resin) into the mold was done in slow manner. Brisk pouring resulted in the formation of air bubbles thus giving a rough hard surface on the finished products.

Casting of sludge and unsaturated polyester were done at ordinary room conditions. Various sludge to resin mix proportions from 80:20 to 50:50 were made. Observations on the characteristics were likewise made and are summarized in Table 5. The sludge and resin mix at 80 20 have lumps due to insufficient amount of resin, thus, rendering the mixture to be unpourable. The same effect was observed with 70:30 sludge and resin mixture. The 60.40 sludge-resin mix had fluid consistency, no lumps and was pourable. The product formed from this mixture had smooth surface. The 50:50 mixture of sludge and resin had the same characteristics as that of 60:40 mix except that it was less viscous.

The casting of sludge and unsaturated polyester was cured rapidly through the catalytic action of methylethylketone peroxide. The curing period was about an hour. During this time, the temperature of the mixture rose rapidly to about 45°C which accelerated the curing action. Cross-linking, the main polymerization reaction, during curing was achieved by polymer molecules interconnection with primary covalent bonds occuring at unsaturated sites.

Stability Test

There was no marked difference between the thermosets with sludge particles stored at room temperature with that of samples exposed at outdoor conditions except for the decrease in the weight of samples (see Tables 6 & 7). This might be primarily due to the

fact that thermoset is composed of a network of chains held together by primary covalent cross links. Therefore, its response to temperature is different than that of a thermoplastic. Thermoset, once cross-linked, would not be easily affected by varying temperature conditions.

Leaching Test

The acidic solution used for determining leachability of untreated and thermoset encapsulated sludge was subjected to heavy metal analysis to determine amount of Cu leached. Results of the leaching test is summarized in Table 8. Undoubtedly, the untreated sludge gave a high copper leached after 14 days, 262 mg/L, while the sludge encapsulated by thermosetting material leached out 40.13 mg/L copper. After 28 days, the copper leached from untreated sludge was 353.1 mg/L and 68.37 mg/L from the thermoset-encapsulated sludge. The acid solution from untreated sludge gave high concentration of copper leached, 447.2 mg/L, after 56 days while the thermoset-encapsulated sludge had 102.5 mg/L. Based on results obtained, shown graphically in Figure 13, it can be concluded that thermoplastics offered superior encapsulating properties for heavy metal bearing sludge than thermosets. This could be probably due to the formation of water molecules during condensation polymerization in the casting of thermosets that facilitated the instability of heavy metal ions. The unstable heavy metal ions were vulnerable to the mobilization or dissolution mechanism when the encapsulated waste was contacted by leaching solution which resulted in a net transfer, or leaching of contaminants into the solution. The nonhomogeneity of samples and curing time for thermosetting materials also affect leaching test results.

CONCLUSION

Based on the results of the physico-chemical tests conducted on the polymeric encapsulated heavy metals bearing sludge, the thermoplastic encapsulation to as high as 30% was effective while thermoset encapsulation can be done at a maximum sludge concentration of 60%.

The tensile strength test done on thermoplastic encapsulated sludge showed that its elasticity diminished as further confirmed by the results of the impact strength test which showed that incorporation of sludge particles within plastic matrix produced hard plastics. It can be concluded that based on this property, polymer-encapsulated sludge can be used in the processing of plastic products where hardness not elasticity is the main criterion. Morphological analyses of experimental samples revealed that the sludge particles are trapped in the polymer matrix. The type of binding that have taken place is physical binding in the form of encapsulation and, hence, no chemical reaction has occurred.

The thermoset encapsulated sludge in the form of artwares remained stable even at outdoor conditions. This showed that sludge particles were fully coated with unsaturated polyester resin. Compared with thermoplastic encapsulation, thermoset encapsulation

can be done at higher sludge concentration and its processing involved no sophisticated equipment like compounding and moulding machine. However, certain product application, for instance, as packaging products, the use of sophisticated equipment for thermoset encapsulation is inevitable.

The leaching test conducted on encapsulated sludge confirmed that polymeric encapsulation can transform the waste material, specifically heavy metal bearing sludge, into structurally stable products. Although, the results of the leaching tests are not directly applicable to leaching mechanism in the field, this test together with other analyses, like microscopic techniques, can be used as indicator of the possible fate of encapsulated sludge and its environmental impact. The polymeric encapsulation is an important process for managing hazardous wastes and might become increasingly important in the future since many wastes such as those containing heavy metals can not be completely destroyed nor their generation be avoided.

ACKNOWLEDGMENT

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ELEMENTS	PERCENT BY WEIGHT
Copper, Cu	88.47
Tin, Sn	6.86
Iron, Fe	1.49
Phosphorous, P	1.43
Silicon, Si	1.33
Sulfur, S	0.27
Aluminum, Al	0.15

Table 1. Results of the Elemental Analysis.

SAMPLE	THICKNESS (in.)	WIDTH (in.)	LOAD (lb.)	STRESS (lb/in^2)	ELONGATION	STRAIN (in./in.)	MODULUS of ELASTICITY (lb/in^2)
Virgin PP							
1	0.0980	0.500	188.76	3852.25	0.3550	0.1775	21,702.816
2	0.0945	0.492	196.68	4229.68	0.5890	0.2945	14,362.241
3	0.0905	0.492	193.16	4340.67	0.6420	0.3210	13,522.336
4	0.0945	0.496	190.30	4057.57	0.4820	0.2410	16,836.390
5	0.0945	0.492	192.28	4135.05	0.4890	0.2445	16,912.269
PP with 10% sludge							
1	0.0980	0.496	126.94	2611.93	0.0846	0.0423	61,747.754
2	0.0980	0.496	194.48	4001.65	0.1385	0.0693	57,743.867
3	0.0980	0.500	168.08	3430.20	0.0850	0.0425	80,710.588
4	0.0980	0.496	144.76	2978.60	0.1083	0.0541	55,057.300
5	0.0945	0.492	163.24	3510.54	0.0100	0.0500	70,210.800
PP with 20% sludge							
1	0.0906	0.496	126.28	2812.47	0.0770	0.0385	73,051.168
2	0.0906	0.496	122.76	2734.08	0.0540	0.0270	101,262.220
3	0.0906	0.496	137.28	3067.46	0.0606	0.0303	100,906.270
4	0.0945	0.492	133.10	2862.36	0.0587	0.0294	97,359.183
5	0.0906	0.496	129.14	2876.17	0.0570	0.0285	100,918.24
PP with 30% sludge							
1	0.0980	0.496	146.30	3010.29	0.0709	0.0354	85,036.440
2	0.0980	0.496	94.16	1937.45	0.0287	0.0144	134,545.130
3	0.0980	0.496	157.08	3232.10	0.882	0.0441	73,290.249
4	0.0980	0.496	112.86	2322.22	0.0492	0.0246	94,399.186
5	0.0980	0.496	122.40	2518.50	0.0456	0.0228	110,460.520

Table 2. Tensile Test, Strain and Modulus of Elasticity of Thermoplastic Encapsulated Sludge.

SAMPLE	WIDTH (mm)	READING (ft-lbf)	CORRECTION FACTOR (ft-lbf)	CORRECTED READING (ft-lbf)	IMPACT STRENGHT (ft-lbf/mm)	TYPE OF BREAK
Polypropylene	12.75	0.125	0.07	0.055	0.00431	complete
	12.71	0.120	0.07	0.05	0.00393	complete
	12.75	0.120	0.07	0.05	0.00392	complete
	12.76	0.120	0.07	0.05	0.00392	complete
	12.80	0.125	0.07	0.055	0.00430	complete
10% sludge	12.85	0.125	0.07	0.055	0.00428	complete
	12.94	0.120	0.07	0.05	0.00386	complete
	12.80	0.125	0.07	0.055	0.00430	complete
	12.80	0.120	0 07	0.05	0.00390	complete
	12.80	0.120	0.07	0.05	0.00390	complete
20% sludge	13 10	0.130	0.07	0.06	0.00458	complete
	12 66	0.120	0 07	0.05	0.00395	complete
	12.75	0 120	0.07	0.05	0.00392	complete
	12 89	0.120	0 07	0.05	0.00388	complete
	12.85	0.120	0.07	0.05	0.00389	complete
30% sludge	12 59	0.120	0.07	0.05	0.00397	complete
	12 70	0.120	0.07	0.05	0.00394	complete
	12 84	0.120	0.07	0.05	0.00389	complete
	12.85	0.130	0 07	0.06	0.00467	complete
	12.82	0.120	0.07	0 05	0.00390	complete

Table 3. Results of Impact Test

SAMPLE	AVERAGE IMPACT STRENGTH	
	ft-lbf/mm	J/m
Polypropylene	0.004076	5.5263
Thermoplastic with 10% sludge	0.004042	5.4802
Thermoplastic with 20% sludge	0.004044	5.4829
Thermoplastic with 30% sludge	0.004068	5.5154

PP = 0.004076 ft-lbf/mm x 1.355818J/ft-lbf x 1,000 mm/m = 5.5263 J/m
10% sludge = 0.004042 ft-lbf/mm x 1.355818 J/ft-lbf x 1,000 mm/m = 5 4802 J/m
20% sludge = 0.004044 ft-lbf/mm x 1.355818 J/ft-lbf x 1,000 mm/m = 5.4892 J/m
30% sludge = 0.004068 ft-lbf/mm x 1.355818 J/ft-lbf x 1,000 mm/m = 5.5154 J/m

Table 4. Average Izod impact strength.

PROPORTION		OBSERVATION
RESIN	SLUDGE	
20 parts	80 parts	mixture had lumps unpourable
30 parts	70 parts	hard to pour mixture had lumps
40 parts	60 parts	mixture had fluid consistency pourable products had smooth surface
50 parts	50 parts	pourable products had smooth surface consistency was more fluid

Table 5. Sludge-Resin Mix For Casting

AGE (days)	WEIGHT (g)	TIME	OBSERVATION
2	152	8:10 AM	no visible change
5	152	3:05 PM	no visible change
7	151.8	1:05 PM	no visible change
9	152	4:00 PM	no visible change
12	151.7	3:05 PM	no visible change
15	151.8	1:30 PM	no visible change
17	151.8	9:30 AM	no visible change
19	151.8	8:00 AM	no visible change
20	151.8	10:30 AM	no visible change
22	151.8	10:00 AM	no visible change
24	151.7	8:00 AM	no visible change
27	151.7	3:00 PM	no visible change
30	151.7	2:00 PM	no visible change

Table 6. Stability Test of Thermoset Encapsulated Sludge (Stored at Air Conditioned Room).

AGE (days)	WEIGHT (g)	TIME	WEATHER	OBSERVATIONS
2	155.00	8:00 AM	sunny	no visible change
5	155.00	3:00 PM	sunny	no visible change
7	155.00	1:00 PM	sunny	no visible change
9	154.00	4:10 PM	sunny	no visible change
12	154.20	3:00 PM	sunny	no visible change
15	154.00	1:20 PM	sunny	no visible change
17	153.90	9:00 AM	sunny	no visible change
19	158.60	7:45 AM	stormy	sample is wet
20	155.60	10:20 AM	cloudy	no visible change
22	154.00	9:45 AM	sunny	no visible change
24	153.80	8:30 AM	sunny	no visible change
27	153.60	3:20 PM	sunny	no visible change
30	153.80	2:30 PM	sunny	no visible change

Table 7. Stability Test of thermoset Encapsulated Sludge (Stored at Outdoor conditions).

Sample	Weight (g)	Volume of leaching solution (mL)	Copper leached (mg/L)		
			14 days	28 days	56 days
Thermoplastic					
10% sludge	8.3	166	1.56	1.83	2.3
20% sludge	9.0	180	1.96	2.9	3.8
30% sludge	7.0	140	1.25	3.25	7.8
Thermoset	152	3,040	40.13	68.37	102.5
Untreated sludge	27	540	262	353.1	444.2

Table 8. Results of Leaching Test

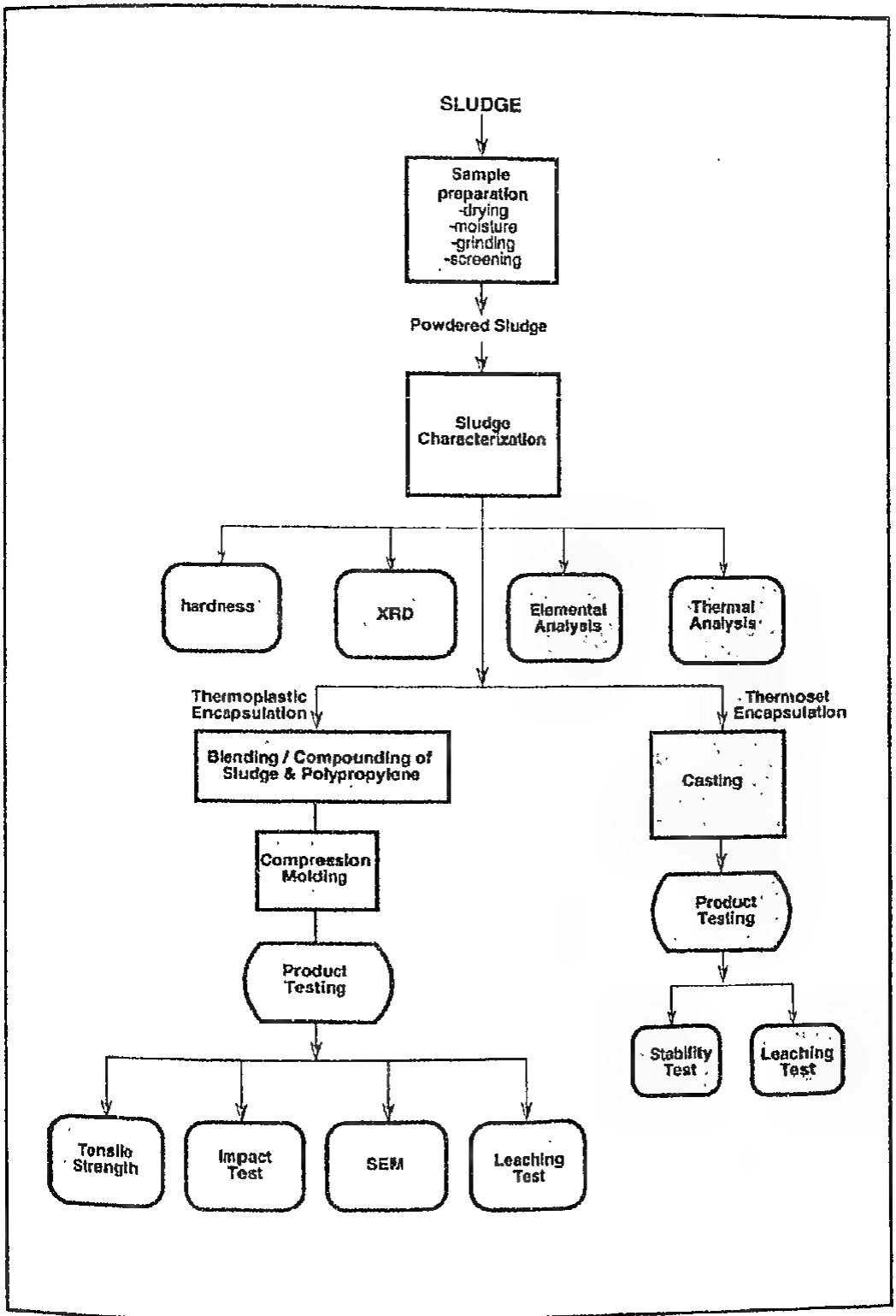


Figure 1. Experimental Design for Polymeric Encapsulation

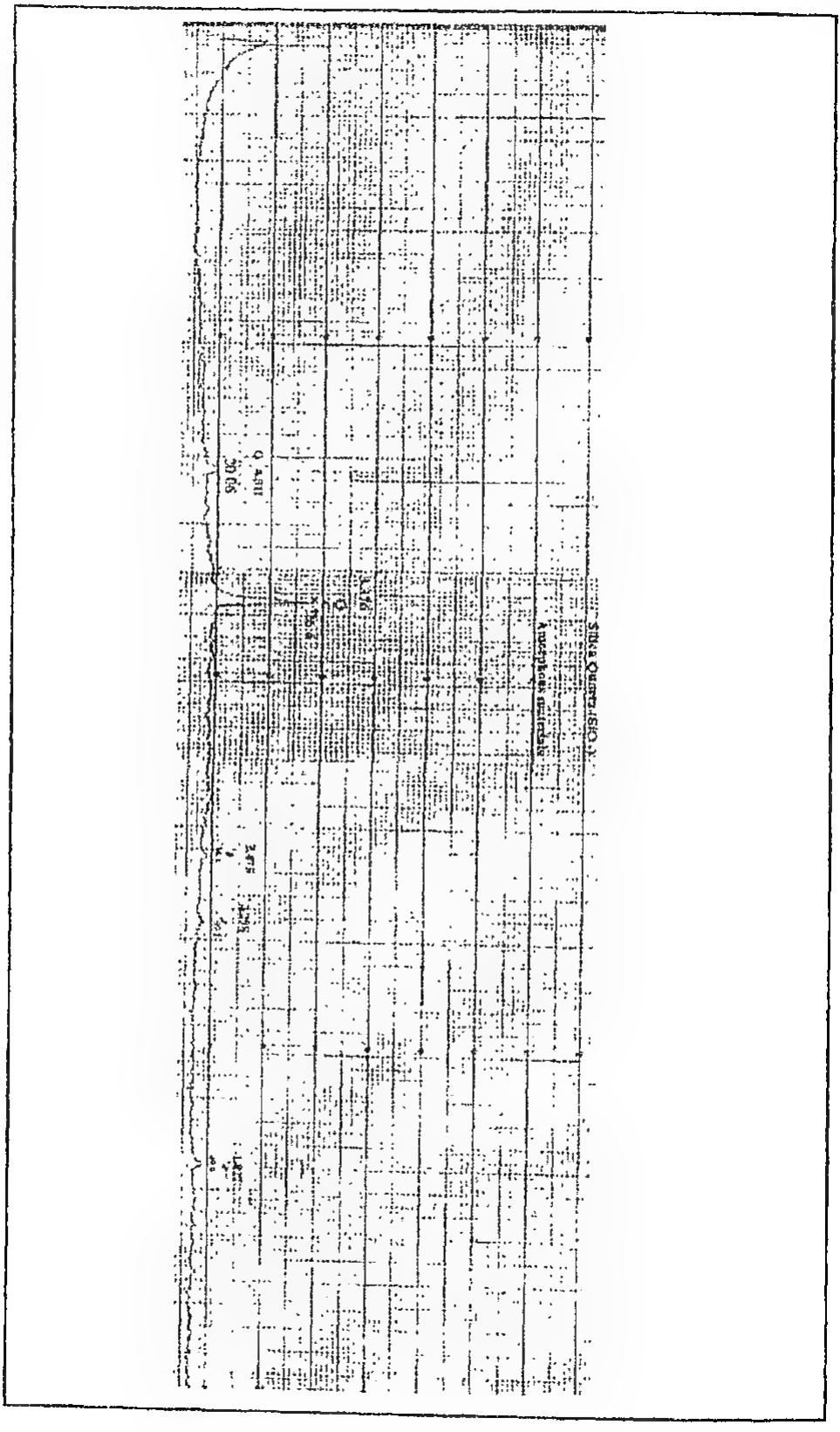


Figure 2. Results of X-Ray Diffraction Analysis.

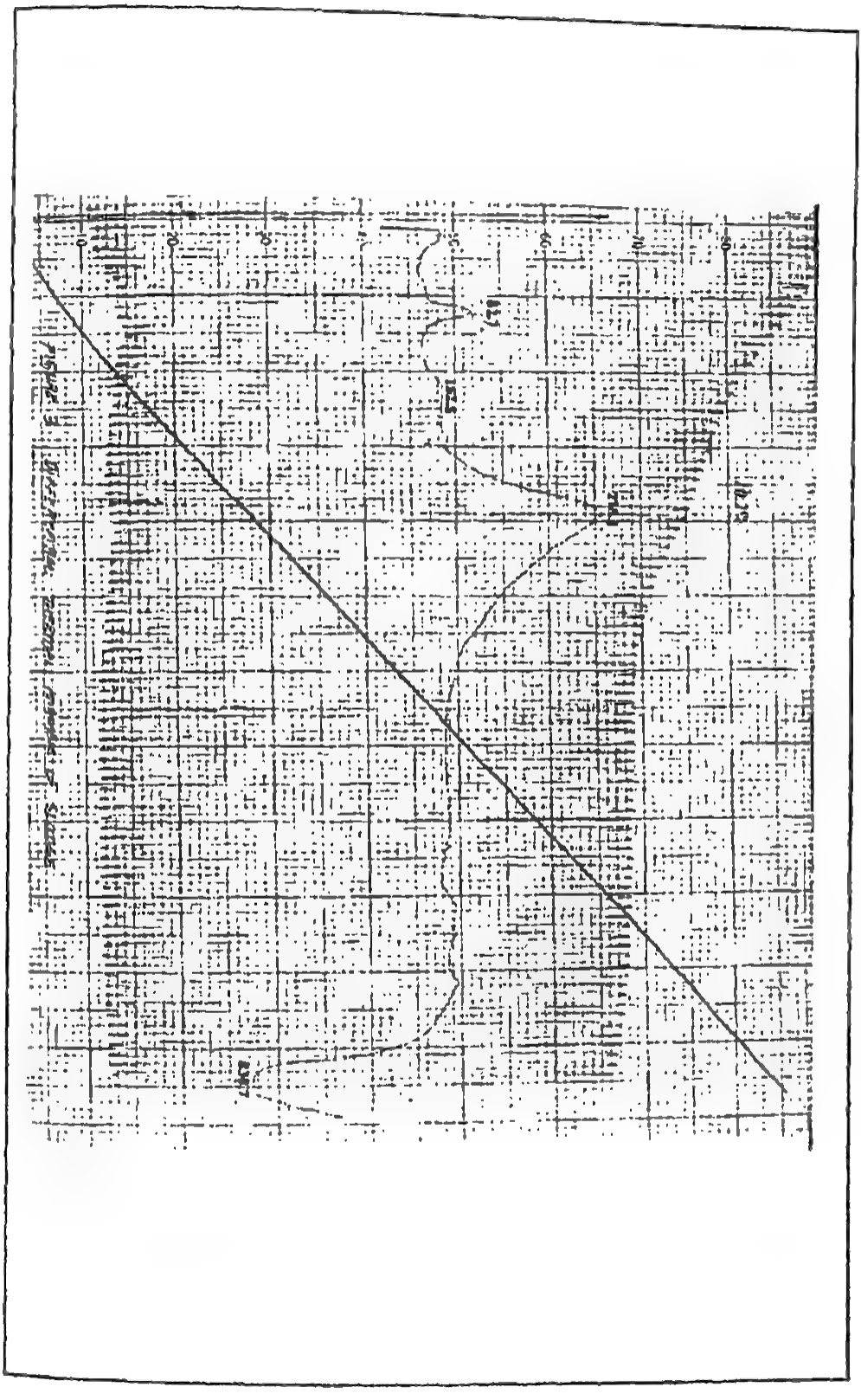


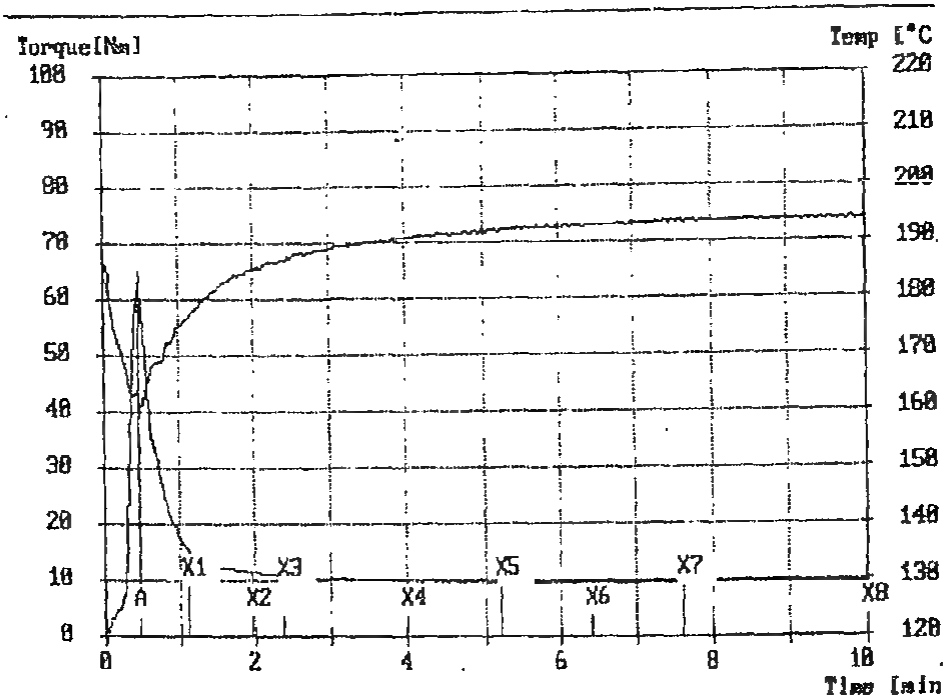
Figure 3 Differential Thermal Analysis of Sludge

BRABENDER

Data-Processing PLASTI-CORDER PL2000 and Mixer Measuring Head
Semi-Automatic Universal Evaluation

Test Conditions

Order	: PP	Mixer Temp.	:	190	°C
Operator	: BARBIE	Speed	:	50	1/min
Test Date	: 27. Sep '94	Meas. Range	:	100	Nm
PL Type	: 2000-3	Zero Suppr.	:	0	%
Mixer Type	: W 50	Damping	:	1	
Load. Chute	: pneumatic	Test Time	:	20.0	min
Sample	: PP	Sample Weight	:	40.00	g
Additive	:	Code Number	:	1	
		Start Temp.	:	186	°C



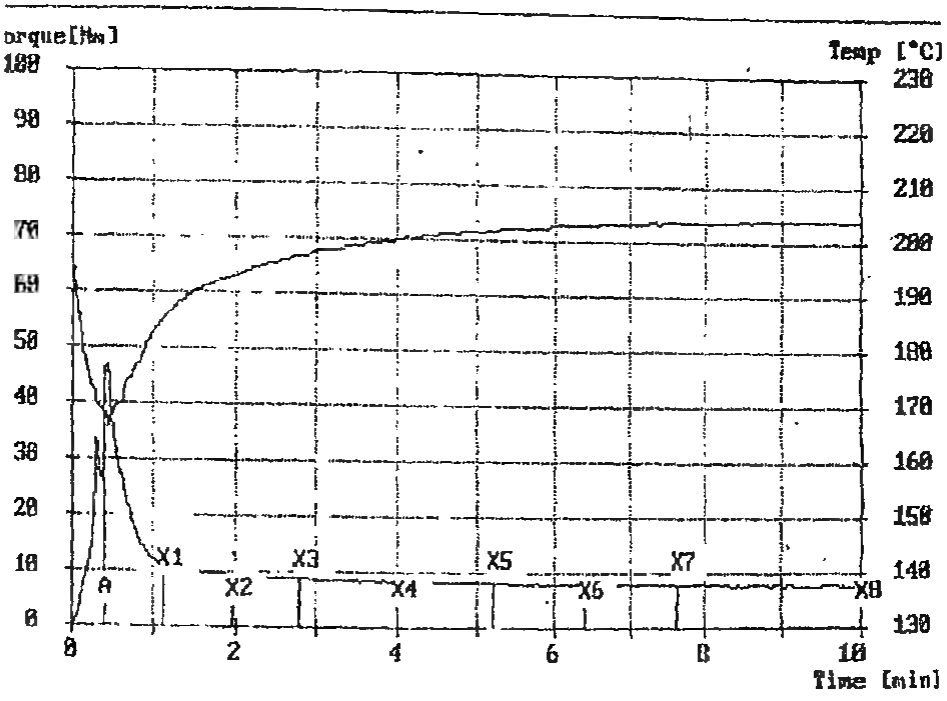
	Time 0 to X	Time X to X	Torque Nm at X	Temp. °C at X	Energy kNm 0 to X	Energy kNm X to X
A	00:00:28	00:00:28	65.0	161	3.4	3.4
X1	00:01:06	00:00:38	15.5	176	9.0	5.6
X2	00:01:58	00:00:52	11.5	185	12.4	3.4
X3	00:02:22	00:00:24	10.8	187	13.8	1.4
X4	00:04:00	00:01:38	9.9	191	19.1	5.3
X5	00:05:12	00:01:12	9.[illegible]	192	22.8	3.7
X6	00:06:24	00:01:12	9.8	193	26.5	3.7
X7	00:07:36	00:01:12	9.6	193	30.1	3.6
X8	00:10:00	00:02:24	9.5	194	37.4	7.2

Figure 4. Plastogram of Pure Polypropylene (PP).

BRABENDER

Data-Processing PLASTI-CORDER PL2000 and Mixer Measuring Head
Semi-Automatic Universal Evaluation

st Conditions					
der	: SLUDGE		Mixer Temp.	:	200 °C
erator	: GARBIE		Speed	:	50 1/min
st Date	: 27. Sep '94		Meas. Range	:	100 Nm
Type	: 2000-3		Zero Suppr.	:	0 %
xer Type	: W 50		Damping	:	1
ad, Chute	: PNEUMATIC		Test Time	:	10.0 min
mple	: PP		Sample Weight	:	40.00 g
ditive	: 10%sludge2		Code Number	:	-
			Start Temp.	:	195 °C



	Time 0 to X	Time X to X	Torque Nm at X	Temp. °C at X	Energy kNm 0 to X	Energy kNm X to X
1	00:00:24	00:00:24	46.2	169	2.1	2.1
2	00:01:08	00:00:44	11.0	185	6.9	4.7
3	00:01:58	00:00:50	9.4	193	9.5	2.6
4	00:02:48	00:00:50	8.8	197	11.8	2.4
5	00:04:00	00:01:12	8.3	200	15.1	3.2
6	00:05:12	00:01:12	8.0	202	18.1	3.1
7	00:06:24	00:01:12	7.9	203	21.2	3.0
8	00:07:36	00:01:12	7.8	204	24.1	3.0
9	00:10:00	00:02:24	7.7	204	29.9	5.8

Figure 6. Plastogram of PP with 10% Sludge

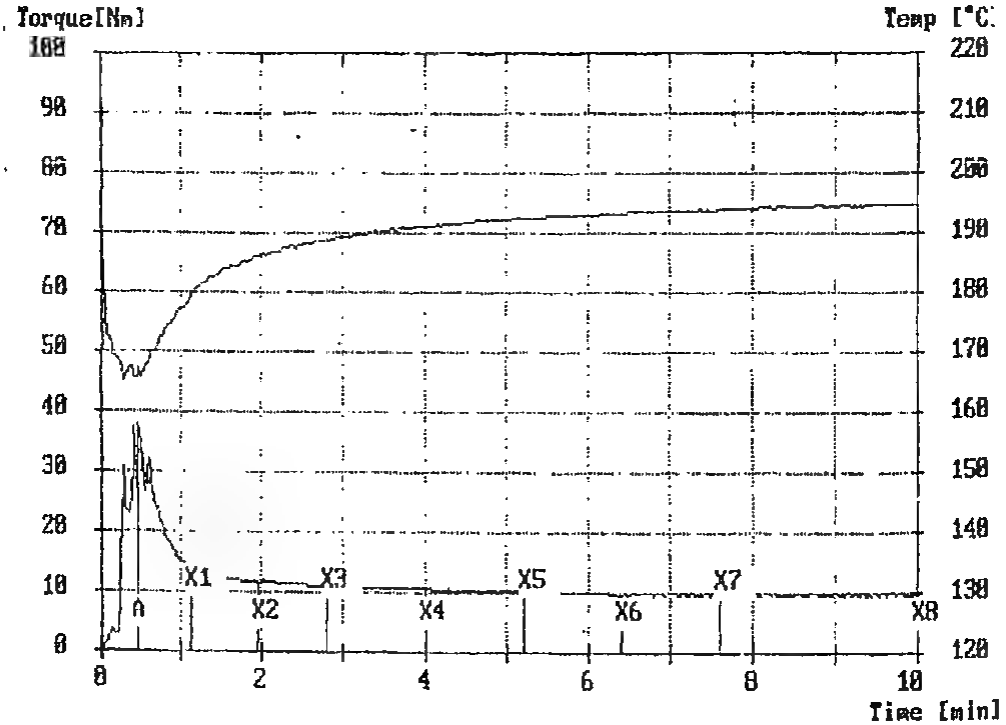
Figure 5. Plastogram of PP with 10% Sludge.

BRABENDER

Data-Processing PLASTI-CORDER PL2000 and Mixer Measuring Head
Semi-Automatic Universal Evaluation

Test Conditions

Order	: SLUDGE		Mixer Temp.	:	190 °C
Operator	: BARBIE		Speed	:	50 1/min
Test Date	: 27. Sep '94		Meas. Range	:	100 Nm
PL Type	: 2000-3		Zero Suppr.	:	0 %
Mixer Type	: W 50		Damping	:	1
Load. Chute	: PNEUMATIC		Test Time	:	20.0 min
Sample	: PP		Sample Weight	:	40.00 g
Additive	: 20% SLUDGE		Code Number	:	-
			Start Temp.	:	187 °C



	Time 0 to X	Time X to X	Torque Nm at X	Temp. °C at X	Energy kNm 0 to X	Energy kNm X to X
A	00:00:28	00:00:28	37.8	167	2.5	2.5
X1	00:01:08	00:00:40	13.9	180	6.9	4.4
X2	00:01:58	00:00:50	11.8	186	10.1	3.3
X3	00:02:48	00:00:50	10.9	189	13.1	3.0
X4	00:04:00	00:01:12	10.5	191	17.1	4.0
X5	00:05:12	00:01:12	10.1	193	21.0	3.9
X6	00:06:24	00:01:12	9.7	193	24.8	3.8
X7	00:07:36	00:01:12	10.0	194	28.5	3.7
X8	00:10:00	00:02:24	9.8	195	36.0	7.4

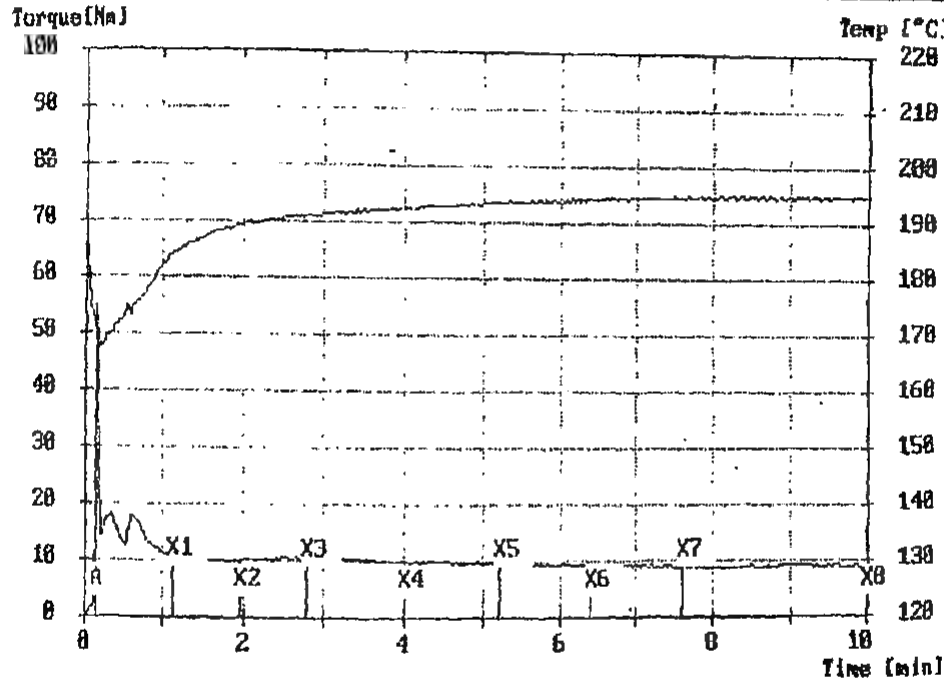
Figure 6. Plastogram of PP with 20% Sludge.

BRABENDER 2

Data-Processing PLASTI-CORDER PL2000 and Mixer Measuring Head
Semi-Automatic Universal Evaluation

Test Conditions

Order	: SLUDGE	Mixer Temp.	:	190	°C
Operator	: BARBIE	Speed	:	50	1/min
Test Date	: 27. Sep '94	Meas. Range	:	100	Nm
PL Type	: 2000-3	Zero Suppr.	:	0	%
Mixer Type	: W 50	Damping	:	1	
Load. Chute	: PNEUMATIC	Test Time	:	20.0	min
Sample	: PP	Sample Weight	:	40.00	g
Additive	: 30% SLUDGE	Code Number	:	-	
		Start Temp.	:	187	°C



	Time 0 to X	Time X to X	Torque Nm at X	Temp. °C at X	Energy kNm 0 to X	Energy kNm X to X
A	00:00:10	00:00:10	55.1	170	0.7	0.7
X1	00:01:08	00:00:58	10.9	184	5.1	4.4
X2	00:01:58	00:00:50	10.2	189	7.8	2.7
X3	00:02:48	00:00:50	10.5	191	10.5	2.7
X4	00:04:00	00:01:12	10.0	192	14.4	3.9
X5	00:05:12	00:01:12	9.7	194	18.1	3.7
X6	00:06:24	00:01:12	9.3	194	21.6	3.6
X7	00:07:36	00:01:12	9.1	195	25.1	3.5
X8	00:10:00	00:02:24	8.9	196	31.9	6.8

Figure 7. Plastogram of PP with 30% Sludge.

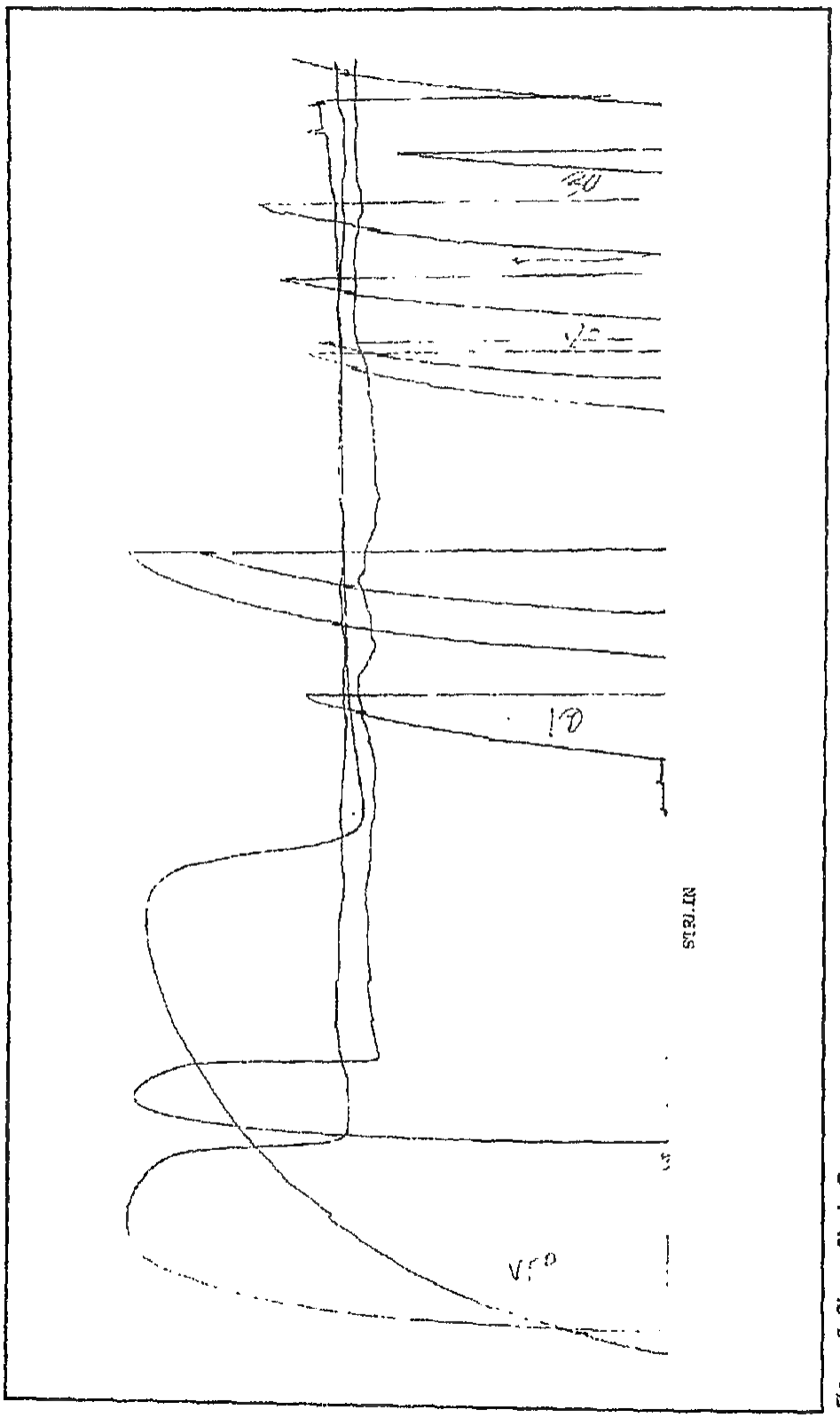


Figure 8. Stress-Strain Curve.

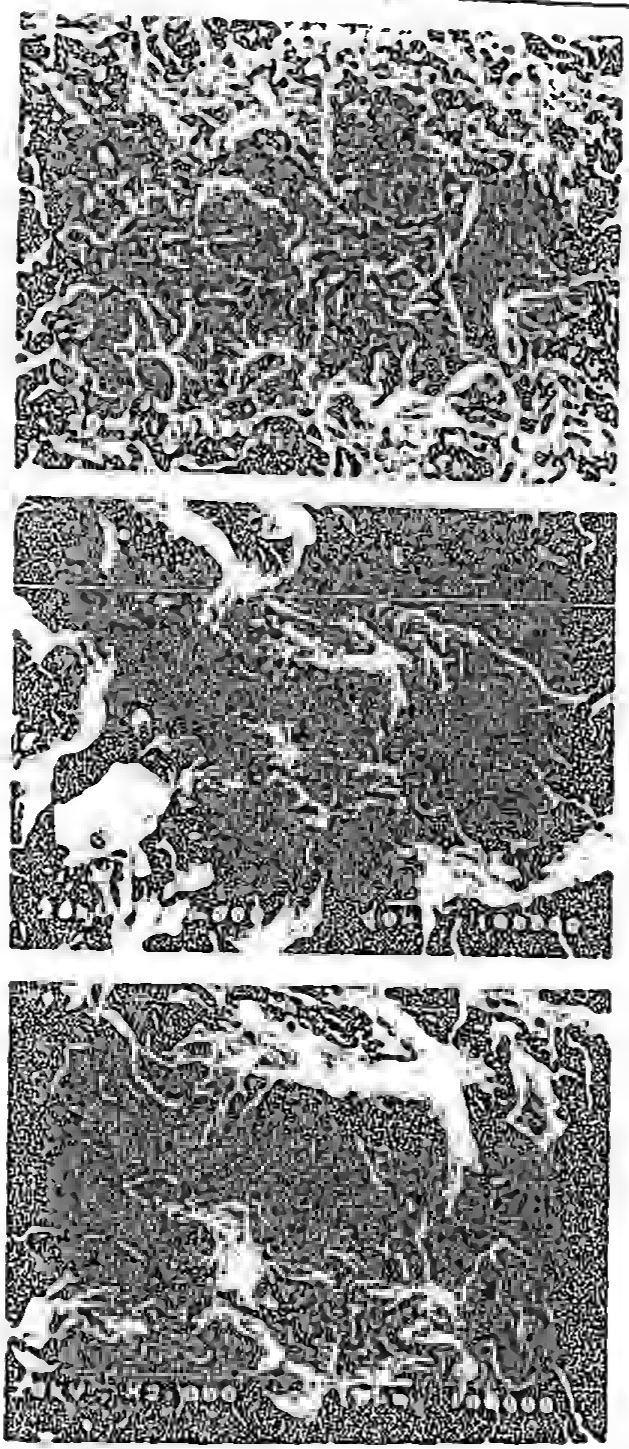


Figure 9. Micrographs of Pure Polypropylene.

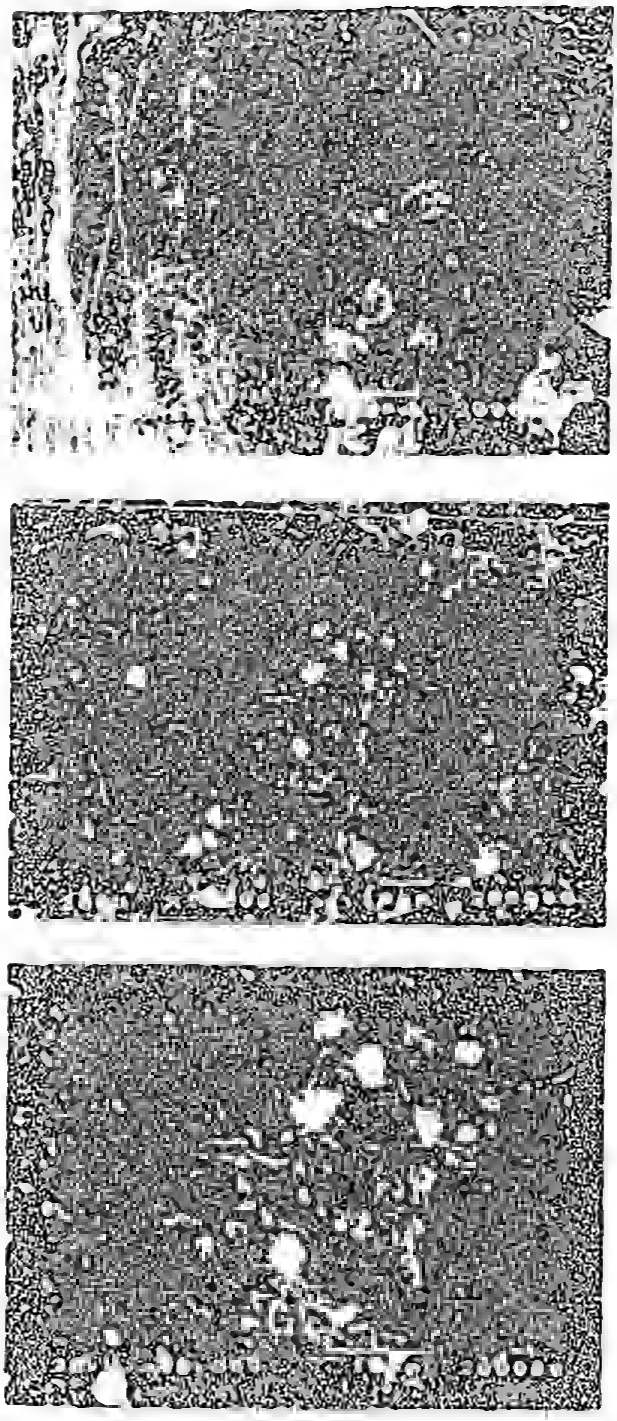


Figure 10. Micrographs of Polypropylene with 10% Sludge.

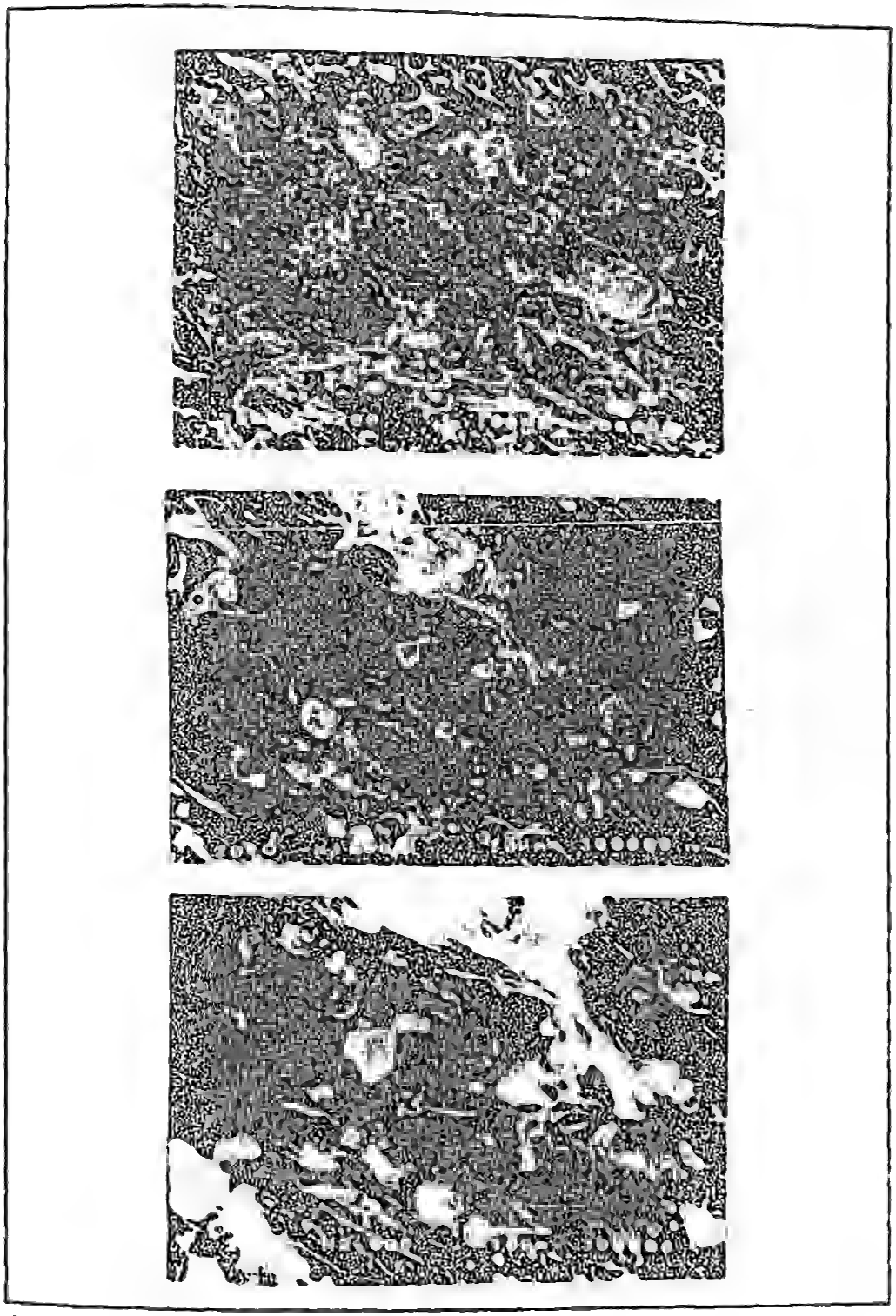


Figure 11. Micrographs of Polypropylene with 20% Sludge.

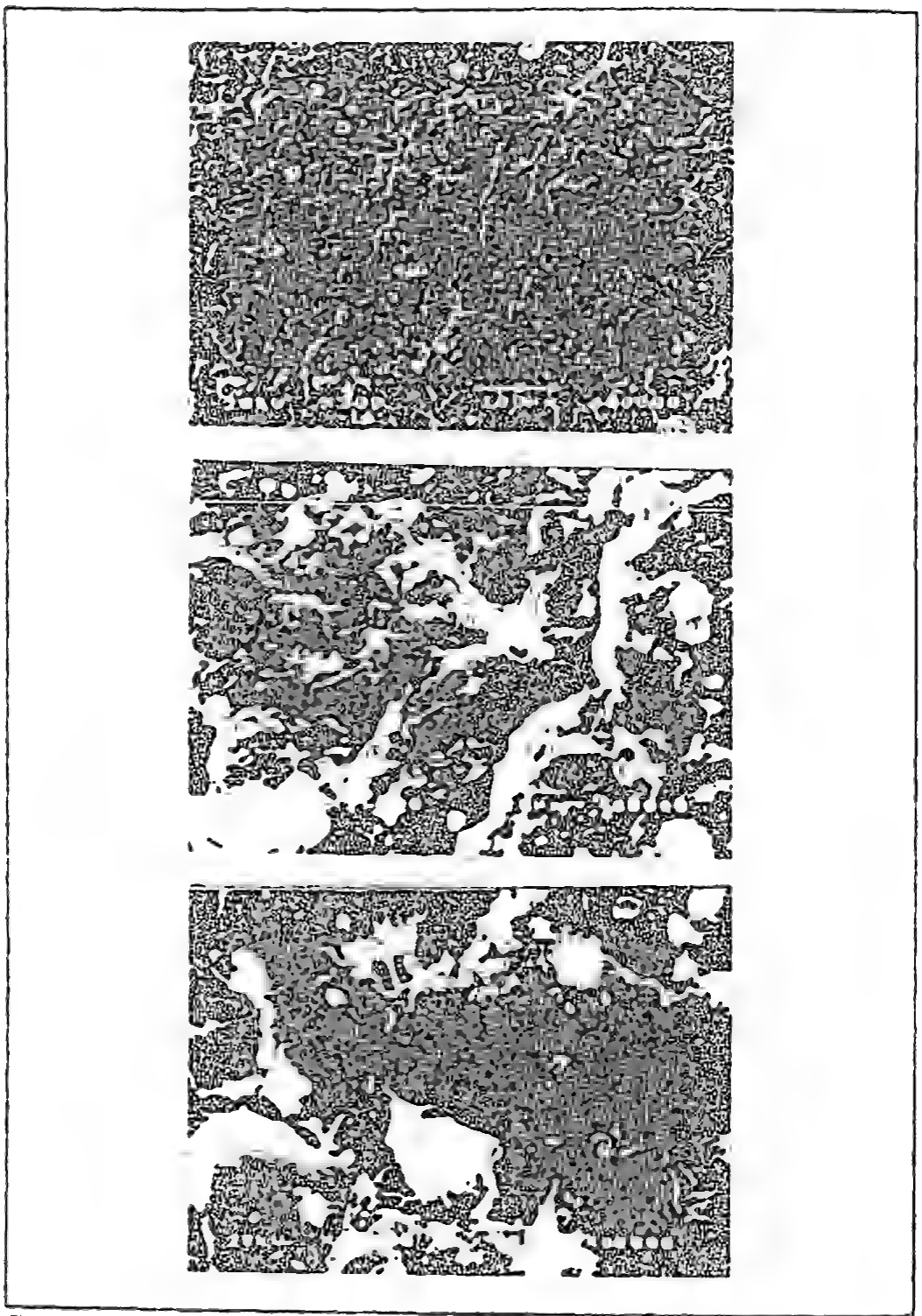


Figure 12. Micrographs of Polypropylene with 30% Sludges.

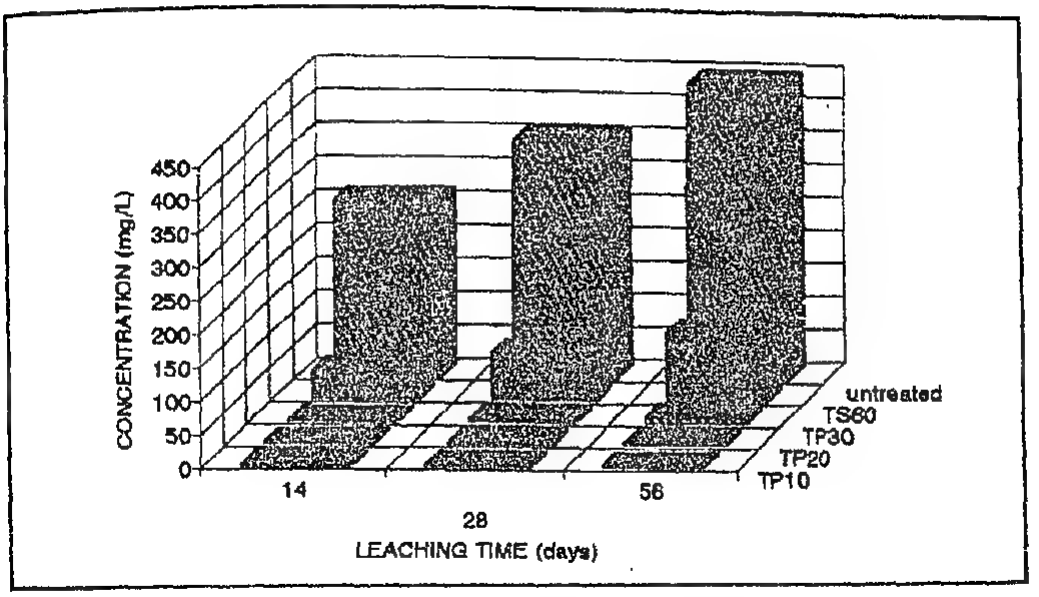


Figure 13. Copper Leached afte 14, 28 & 56 Days

Annex A.
Example of U.S. EPA RCRA Hazardous Wastes for which S/S is being evaluated as atreatment Technology.

WASTE CODE	DESCRIPTION OF WASTE	POLLUTANT OF CONCERN FOR S/S
K048-52	dissolved air flotation (DAF) float from the petroleum industry	Chromium, Lead
K061	emission control dust/ sludge from the primary production of steel in electric furnace	Chromium, Lead Cadmium
K046	wastewater treatment sludge from manufacturing formulation and loading of lead-based initiating compound	Lead
F006	metal finishing sludges	Cadmium, Chromium, Lead Nickel, Silver
F012, F019	metal finishing sludges	Cadmium, Chromium, Lead Nickel, Silver
K022	distillation tar	Chromium, Nickel
K001	wood preserving sludges	Lead

Source: US EPA CERI-89-222

Annex B.
Commercial Waste Stabilization Process

VENDOR	PROCESS NAME	INGREDIENTS	REMARKS/COMMENTS
Chemfix, Inc.	Chemfix	cement + soluble silicates	probably does not fix most volatile organics
IU Conversion	Sealosafe Stablex	Silicates	probably does not fix oils, solvents, grease volatile organics
Dravo Lime	Calcilox	glassy slag	designed to fix scubber sludge probably does not fix most volatile organics
Envirotech (Subsidiary of Chemfix)	Envirotech	cement and silicates	U.S. Patent 3,837,872
Velsicol	Velsicol	fly ash, scrubber sludge and cement	claims to stabilize organics; not specific
Stabitrol	Terra-Tite	cement	probably does not fix most volatile organics
TRW Systems	-	1. cement plaster and	does not fix volatile organics
		2. polybuta-diene resin	may work for organics very costly
U.S. Gypsum	Envirostone	Gypsum	does not fix volatile organics

SOURCE: U.S. EPA CERI-89-222

Annex C.
MOHS Scale of Hardness

SCALE	CLASSIFICATION
1	TALC
2	GYPSUM
3	CALCITE
4	FLUORITE
5	APATITE
6	ORTHOCLASE
7	QUARTZ
8	TOPAZ
9	CORUNDUM
10	DIAMOND

SOURCE: Dana's Manual of Mineralogy

EXTRACTION OF OCTOCHAETID EARTHWORMS, *EUTYPHOEUS GAMMIEI* USING AN AQUEOUS EXTRACT OF *POLYGONUM HYDROPIPER LINN*, WITH A COMPARISON OF OTHER CHEMICAL METHODS FOR ESTIMATING EARTHWORM POPULATIONS

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ABSTRACT

Treatment of soil, infested with fresh castings of earthworms, with a dilute aqueous extract of Polygonum hydropiper (500 g crushed plant material mixed with 10 liters of water) quickly exerts expulsion of about 23 individuals of Eutyphoeus gammiei per sq.m. from the burrows or tunnels within 5 minutes following inundation of the area with the solution. These extract induced expelled worms thrive well when immediate fresh water bath (at least with 2-3 changes for 15 minutes) was given. When compared with other chemical methods of earthworm extraction, it has been observed that plant extract method was efficacious as formalin method in retrieving adequate number of earthworm, Eutyphoeus gammiei from soil.

INTRODUCTION

Earthworms are considered to be the most beneficial organisms to agriculture and are called "nature's ploughman". However, they are found to cause some unwholesome activities so as to produce ugly castings all over the dwelling places, uprooting of seedlings and harbouring disease-causing organisms. Early practice of application of salt water over the soil to extract worms for subsequent angling (Walton and Cotton, 1976) or their annihilation over the lawns (Hudson, 1919) is on record. Indeed, several methods for the extraction of these worms are known, viz., (i) hand sorting, (ii) formalin method (Raw 1959, Dash and Patra, 1972), (iii) Permanganate method (Evans and Guild, 1947; Dash and Patra, 1972) , (iv) Salt (sodium chloride) water treatment (Ali et al., 1973), (v) electrical method (Satchell, 1955) and so on. However, none of the methods is suitable for all the species preferring to live in varied habitats (Satchell, 1967). Careful hand sorting under a good light was considered to be the most accurate method but such method is laborious and the efficacy depends on the density of the root-mat as well as the clay content of the soil (Springett, 1981) in question. Electrical extraction technique is also disadvantageous because the volume of the soil treated is indefinite and the earthworms close to the electrode are very likely to succumb (Kale, 1988).

In Tripura, aqueous extracts of the plant *Polygonum hydropiper* are often sprayed over the courtyard soil to minimise the surface activities (viz.,production of unpleasant

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castings in dwelling places etc.) of several species of earthworms besides using them as bait for angling. Chemical analysis of *P. hydropiper* and biological activity test of its isolated chemical compounds showed that polypiperic acid (a nonsequiterpene acid and acyglucosyl sterol as the main active factors present in the plant extract that have toxic effects on earthworms, *E. gammiei* (Choudhuri et al., 1994). Following application of the two biologically active compounds of P. hydropiper i.e. saturated solution of 0.2% acylglucosyl sterol (solvent:water), as well as 0.2 % polypiper acid (solvent:ethanol-water (1:9) earthworms exhibit instant grotesque behavioral and physiological changes:erratic and escaping movement, exudation of profuse mucous and coelomic fluid coupled with rapid defecation, constriction of different parts of the body etc. (Chaudhuri et al 1994). Chaudhuri and Nanda (1990)also reported similar type of reaction in earthworm (Eutyphoeus sp.) following spraying with crude aqueous extract of *P.hydropiper*. Keeping these findings in mind, we therefore undertook an investigation that deals with (i) method of preparation, standardisation and subsequent application of the plant extract for the collection of an adequate number of giant earthworms, *Eutyphoeus gammiei* (most common and dominant species in Agartala) per unit area of the experimental field and (ii) assessment for the efficacy of the plant *(Polygonum hydropiper)* extract method in contrast with other conventional methods adopted for worm extraction.

MATERIALS AND METHODS

Different chemical methods were used for extraction of octochaetid worm, *Eutyphoeus gammiei*. In an aerable orchard (location: Shibnagar, Agartala) infested with fresh castings of twenty quadrat areas (each quadrat comprising of 1 sq m), considered to be as experimental plot, were selected for chemical treatment during the span of October to November, 1993. Five quadrats were treated with each chemical. Prior to treatments, all the prevailng castings were removed from the experimental plots in order to allow easy penetration of chemicals inside the soil. Each of the trials was replicated twice.

Plant (Polygonum hydropiper) extract method

The fresh plants, Polygonum hydropiper, (local name; Bistakali, family: Polygonaceae, distributuion: Tripura, Assam and North-Eastern states of India, Madras, Bangladesh etc.) were collected from Agartala, Tripura and identified as per scientific procedure. Aerial part (above 500g) of the fresh plant material was crushed in mortar and pestle. The pressed material was mixed with 10 liters of water and stirred well. The mixture was then allowed to settle for about 5 minutes and filtered through a clean coarse cotton mesh. Half of the collected filtrate was applied to quadrat of 1 sq. m. A second course of application was made when the initial treatment did not yield any effect i.e., absence of infestation of worms over the plot surface.

Formalin method

50 ml. of 40% formalin was mixed with 10 litres of water and the solution was applied to 1 sq. m. area.

Permanganate method

Similar to the preceding formalin method, a solution of 15g potassium permanganate in 10 litres of water was used for each application.

Salt (Sodium chloride) water method

A solution was made by mixing 400g sodium chloride with 10 litres of water. This mixture was applied to each quadrat as described above.

RESULTS AND DISCUSSION

Following inundation of the experimental plot in question with the solution of plant (Polygonum hydropiper) extract, abundant number of worms wriggled out from the subsoil burrows (Fig. 1). Indeed, the expelled worms thrived well when immediate fresh water bath at least 2-3 changes for 15 minutes was given.

Collection of the vermes were made on the basis of their sizes which eventually interrelated with their aging. The large mature specimens ranging from 300-350 mm in length are considered to be matured while small to medium (80-200 mm) belonged under the juvenile or immature individuals.

Table I- IV demonstrates the effectiveness of different chemical methods for earthworms (Eutyphoeus gammiei) extraction.

Considering the quantum of expulsion of *Eutyphoeus gammiei* through the use of various chemical treatments, it is obvious that extracts from Polygonum hydropiper and formalin had almost similar effects with reference to the extraction of worms. In contrast, treatment with potassium permanganate proves to be not that suitable for the extraction of mature worms despite the fact that it proves to be the most effective for the extraction of abundant number of worms. Eutyphoeus gammiei obtained through treatment with the plant (P. hydropiper) extract as well as formalin survived provided they were immediately rinsed in water. But worms expelled by potassium permanganate succumbed quickly despite treatment with water. Salt water treatment is less effective for Eutyphoeus gammiei.

The data in Table I- IV clearly reveals that Polygonum hydropiper extract is more suitable for successful extraction of *Eutyphoeus gammiei* not only because it expels large number of individuals in quick succession but also keep the worms healthy following fresh

water bath. Hence, the worms could be successfully cultured in specially prepared culture bed. Besides these, this method is most economic and the crude extract of the plant could be prepared by laymen. Moreover, some of the nuisance activities like uprootings of the seedlings and production of large castings over the dwelling places including courtyards by *Eutyphoeus gammiei* may be monitored through the application of the aqueous extract of *Polygonum hydropiper*.

ACKNOWLEDGMENT

The authors express their sincere thanks to Dr. B.P. Haldar, Zoological Survey of India , Calcutta for identification of earthworm and Dr. Prantosh Roy, Women's College, Agartala, for identification of the plant

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Table 1. Average number of worms (E. gammiei) extracted from a quadrat of 1sq.m. in an areable orchard.

	Mean No. of mature worms extracted by chemical /m^2	Mean No. of immature worms extracted by chemical /m^2	Mean No. of orms extracted by chemical /m^2
Plant (Polygonum hydropiper) extract	23	12 medium - 8 small - 4	35
Formalin solution	22	8 medium 6 small-2	30
Pottasium permanganate solution	12	20 medium - 6 small - 14	32
Salt (SodiumChloride) water	8	-	8

Table 2. Showing the rate of survivality of the extracted mature worms (E. gammiei) undergo ing rinsing (15 minutes) in fresh water following chemical treatments.

Treatment	Mean No. of worms extracted by chemical (m^2)	Mean No. of worms survived after chemical treatment (after 12 hrs.)	Mean No.of worms survived following rinsing in water (after 48 hrs.)	Rate of survival of worms following rinsing in water
Plant (Polygonum hydropiper) extract	23	-	20	87%
Formalin Solution	22	-	19	86.4%
Potassium permanganate Solution	12	-	-	-
Salt (Sodium chloride) water	8	-	6	75%

Table 3. Showing the number of dead and live worms (E.gammiei)/sq.m. in the experimental plot (soil) following 24 hrs. of treatment with \ different chemicals.

Treatment	Mean No. of dead worms (adult) in the soil	Mean No. of live worms (adult) in the soil
Plant (Polygonum *hydropiper) extract*	2	2
Formalin solution	5	3
Potassium permanganate solution	4	4

Table 4: The working time taken per sample unit for the extraction of E. gammiei by adoption of the following chemical methods.

Treatments	Time per sample unit (minutes)
Plant (*Polygonum hydropiper*) extract	5
Formalin solution	20 - 30
Potassium permanganate	10
Salt water	30 - 35

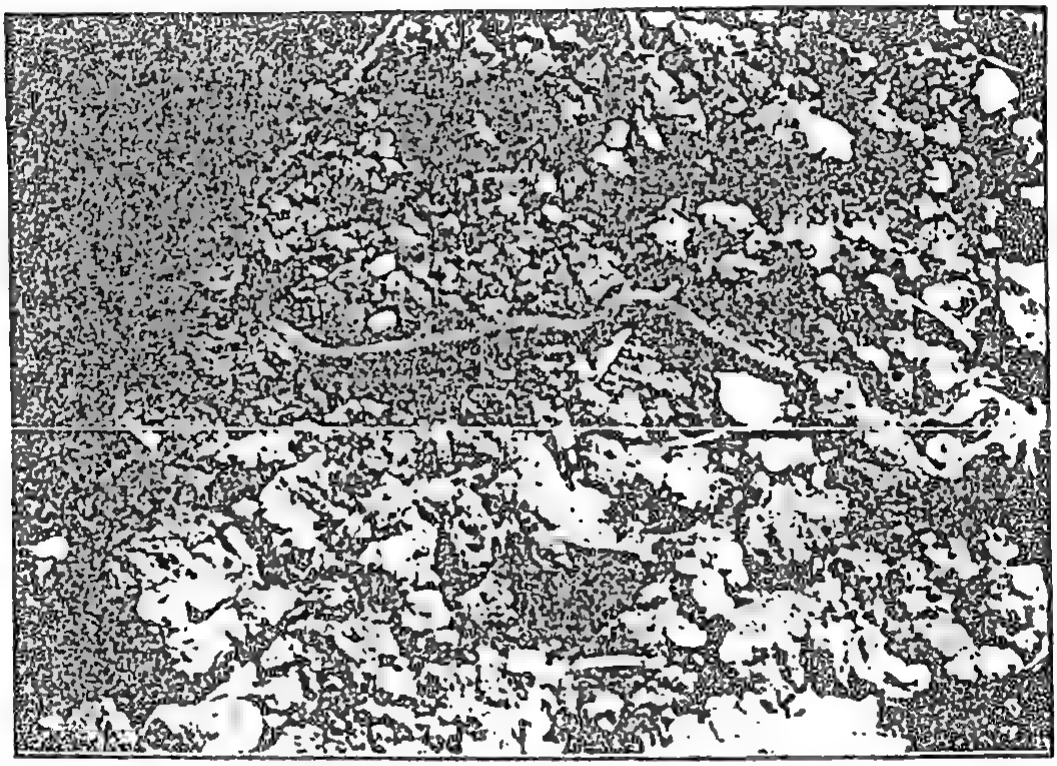

Figure 1. Polygonum hydropiper extract induced emerging worm, Eutyphoeus gammiel from its burrow.

AN ADRENOCORTICOLYTIC AGENT ALTERS THYRO-GONADAL SYSTEM IN THE PIGEON, *COLUMBA LIVIA*

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ABSTRACT

The purpose of the present investigation is to perform "Chemical adrenocortectomy" in pigeon by administration of o,p' - DDD to understand the direct influence of the adrenal cortex on the male gonadal physiology. O,p' -DDD, a selective adrenocorticolytic and antimetastatic agent in humans and dogs, is also used as a pesticide. In contrast to mammals, this drug induces high mortality rate in pigeons. Although o,p'-DDD treatment exerts no perceptible change in the adrenal cortex of pigeon, its degenerative action on the testes and reducing effect on the plasma T_4 level are highly significant. It may be suggested from the above findings that the testicularatrophy is due to o,p'-DDD induced "Chemical thyroidectomy" (through the withdrawal of T_4) in pigeon.

INTRODUCTION

There are quite a few evidences for an involvement of adrenal hormones in the regulation of avian reproductive cycles. Studies on the annual activity of adrenal and gonads have indicated mainly two types of adrenocortical gonadal relationship in birds, namely "parallel type" and "inverse type" of adrenocortical-gonadal relationship were increase and decrease in the adrenocortical tissue activity (Chaturvedi, 1983; Chaturvedi and Thapliyal, 1980; Chaturvedi and Suresh, 1983), whereas in rain quail adrenal and gonadal cycles are inversely correlated (Chaturvedi and Saxena, 1983).

However, direct evidence of the role of adrenal cortex in the control of gonadal physiology can only be obtained by surgical ablation of the adrenocortical tissue. But the complete intermingling of interrenal and medullary tissue in birds (Ghosh, 1980) makes it impossible in discerning the exclusive and separate role played by the adrenal cortex on the gonadal system by specific surgical ablation. Hence, chemical methods may be employed. Gutierrez and Crooke (1980) have used o,p'-DDD as a selective adrenocorticolytic agent to treat metastatic adrenocortical carcinoma in humans without affecting the medulla (Becker and Schumacher, 1975). o,p'-DDD has also been found to induce degenerative changes in the adrenal cortex of dogs (O'Brien, 1967; Breslow et. al., 1990). In addi-

tion, to its therapeutic effects, o,p'-DDD is used as an insecticide (Goodman Gilman et. al., 1990). The aim of the present investigation is to perform "chemical adrenocortectomy" with the help of o,p'-DDD in birds and thereby block the cortical hormones which may directly reflect the regulation of avian gonadal system by the adrenal cortex.

MATERIALS AND METHODS

Twenty eight young adult domestic pigeons *(Columba livia)* were procured from a local bird dealer and housed under uniform laboratory conditions for 7 days with food and water available ad libitum. o,p'-DDD [1,1'-dichloro-2-(o-chlorophenyl) ethane, 'mitotane'; Aldrich, USA] was orally administered to twenty pigeons at a dose of 0.1 mg/bird/day for four days. Sixty percent of the o,p'-DDD treated birds died even before the termination of the experimental period. The remaining birds were left untreated and served as control. On the fifth day, blood samples were collected from the wing vein of the pigeons to estimate plasma T_3, T_4 and testosterone. The birds were then killed by cervical dislocation, their adrenals and testes were dissected out quickly and fixed in suitable fixatives for cytological procedures. Simultaneously, adrenal glands of pigeons were removed and processed for biochemical quantitation of adrenomedullary catecholamines and corticosterone following the spectrophotofluorometric methods of Laverty and Taylor (1968) and Glick et. al., (1964) respectively. Radioimmunoassay of T_3 and T_4 was performed using RIA kit supplied by BRIT, Bombay, following the method of Bhandarkar and Pillai (1982). Plasma testosterone was quantitated following RIA technique (Korenman et al., 1978). The kit was supplied by ICN Biomedicals, Inc., USA. Student's 't' test was used to calculate the significance of difference in hormone concentrations (Snedecor and Cochran, 1967).

RESULTS

Histological: Cortical strands of central zone comprised of tall columnar cells with basal nuclei arranged in a double layer. The finely granular cytoplasm appeared to be slightly vacuolated. o,p'-DDD treatment showed no perceptible change in the cortex. Medullary cells, which are of irregular shape and indefinite arrangement also remained unaffected.

The section of testis of o,p'-DDD treated pigeons were found to be in a completely degenerative condition. The seminiferous tubules were disorganized with a great extent of cellular lysis, while the testis of control birds were found to be in fully active state with successive stages of transformation of the seminiferous epithelium into spermatozoa (Figs. 1 and 2).

Biomedical: Results of the biomedical estimation of adrenomedullary catecholamines, corticosteerone, plasma T_3, T_4 and testosterone contents of o,p'-DDD treated pigeons are summarized in Table 1.

The adrenal content of corticoserone increased up to 45% in o,p'-DDD treated group. If we recall the adrenocortical cytology it may be presumed that the increase of corticosterone content accounts for a 'storage'. The reduction of epinephrine content is only moderately significant. A conspicuous rise in the plasma T_3 level was observed in o,p'-DDD treated pigeons but the T_4 level showed a decrease. Consequently, the $T_3 : T_4$ ratio also increased significantly after the treatment. A trend of lowering of plasma testosterone is evident.

DISCUSSION

The present investigation reveals that o,p'-DDD which acts as drastic adrenocorticolytic agent in mammals (Gutierrez and Crooke, 1980) exhibits no significant effect on the adrenal cortex of a domestic bird, the pigeon. Brief treatment of o,p'-DDD which is proved to be fatal in pigeon, has been reported to have no lethal effect on humans and dogs during prolonged administration even up to 10 months (O'Brien, 1967; Matsumura, 1976; Breslow et. al., 1990).

It has been established by Matsumura (1976) that the antisteroid action of o,p'-DDD suppresses the secretion of corticosteroids and reduces the hormonal effects of the adrenal gland by reducing ACTH in mammals. It is apparent from the present study that the metabolic pathway by which o,p'-DDD is detoxicated in dogs must be drastically different from that of the avian system.

Though adrenal system remains practically non-responsive to o,p'-DDD, degenerative changes of testicular parenchyma are clearly evident. This pesticide (drug) may act either directly on the spermatogenic cells and/or with the spermatids (Jackson, 1972) thereby causing testicular atrophy in birds or through an extra-hypothalamo-hypophysio-adrenocortical pathway in exerting its degenerative effect on the avian testes. The third possibility cannot be totally ruled out, i.e., as o,p'-DDD bears resemblance to its analogue o,p'-DDT, like latter the former may also produce an estrogenic effect on the testis (Bitman et. al., 1968) to inhibit the development of male gonads.

Another important aspects of this study merits discussion. o,p'-DDD treatment appreciably lowers the plasma T_4 level which reflects that the T_4 secretion of the thyroid gland is almost exhausted. We may consider this situation as an instance of "chemical thyroidectomy". This can be correlated with the reports by Thapliyal (1980, 1981). They have observed regression of the gonads of thyroidectomized avian species. However, o,p'-DDD administration does not induce any perceptible change in the plasma T_3 level and an explanation for its static concentration further studies.

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Table 1. Effect of o,p' - DDD on the plasma T_3, T_4, testosterone and adrenal hormones of male domestic pigeon *(Columbia livia)*

Group	Adrenal Corticosterone (ng/100 gm body wt.)	Adrenal Norepinephrine (ng/100 gm body wt.)	Adrenal Epinephrine (ng/100 gm body wt.)	Plasma Testosterone (ng/ml)	Plasma T_3 (ng/ml)	Plasma T_4 (ng/ml)	T_3/T_4 ratio
Control (8)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.22
o,p' DDD Treated (8)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	2.05

[illegible] Mean ± Standard Error
Figures in parentheses [illegible] number of birds used
NS - Not significant

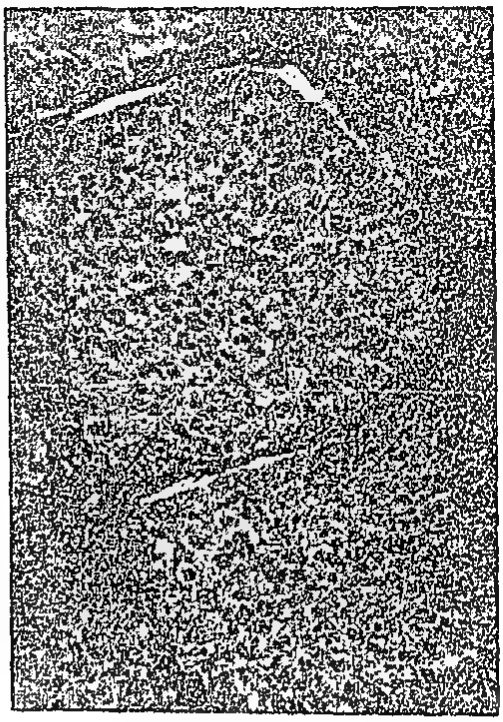

Figure 1. T.S. of testis from control pigeon. Note the seminiferous tubules representing all the germinal cells including the spermatozoa Masson's Trichrome Stain (X 400)

Figuer 2. T.S. of testis from o,p' -DDD treated pigeon. Observe seminiferous tubules are drastically squeezed. Extreme cellular lysis is also noted. Masson's Trichrome Stain. (x 400).

CARBONIC ANHYDRASE: ITS PHYSIOLOGICAL AND EVOLUTIONARY SIGNIFICANCE IN THE MARINE SYMBIONT *PROCHLORON*

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ABSTRACT

The activity of carbonic anhydrase (CA), a photosysthetic enzyme catalyzing the reversible interconversion of HCO_3^- to CO_2, was studied in Prochloron. Measurement revealed that this prokaryotic microalgal symbiont of tropical ascidians exhibits CA activity largely associated with the cell surface. Similar to some chlorophytes, the predominance of extracellular CA and its inhibition increased the K1/2 ($NaHCO_3$) for photosynthesis suggesting that cellular CA in Prochloron is important in facilitating the supply of CO_2 into the cell from HCO^3- which is the form common at high pH values, such as in seawater.

Examination of the effect of sulfonamide inhibitors, acetazolamide and ethoxzolamide, revealed that CA activity of Prochloron is inhibited with I_{50} values of 700 μM and 300 μM, respectively. These I_{50} values bear close resemblance to the measured I_{50} values unicellular cyanobacterium and chloroplasts of green algae and higher plants. Since Prochloron shares characters with both cyanobacteria and green chloroplasts, it could then be place as the possible evolutionary link between the cyanobacteria and chlorophytes.

INTRODUCTION

Prochloron are marine unicellular algae found in symbiotic association with certain tropical didemnid ascidians. They occur in intimate but extracellular association with the ascidians host colony, either attached to the outer surface, embedded in the test or lying in the common cloacal cavity (Cox, 1986). Earlier studies showed that they are prokaryotic, with an ultrastructure resembling that of cyanobacteria or blue-green algae (Lewin, 1975). However, they apparently lack the distinctive photosynthetic pigments, phycobilins, of the cyanobacteria, contain both chlorophylls a and b, and have paired or stacked thylakoids like those of eukaryotic chlorophytes or green algae (Lewin, 1981). Their discovery has generated considerable excitement in the scientific community due to its bearing on theories of the origin eukaryotic chloroplasts, and has prompted much speculation with regard to their unique position in algal phylogeny.

Measurements of photosynthesis revealed the operation of C_4 photosynthetic pathway in Prochloron with 3-phosphoglycerate as the first carbon fixation product (Akazawa et. al., 1978) and ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) as the primary carboxylation enzyme (Berhow and McFadden, 1983). Since CO_2 is the primary substrate for carboxylation by RuBisCO, and not HCO_3-, the commonest form of inorganic carbon in seawater, the enzyme carbonic anhydrase (CA) which analyzes the conversion of HCO_3- to CO_2 was previously assumed to be present in organisms (Alberte, 1989).

In this paper, the actual presence of carbonic anhydrase in Prochloron will be shown and the physiological and evolutionary significance of this enzyme in this particular microalga will be analyzed.

MATERIALS AND METHODS

Collection of ascidian colony and isolation of *Prochloron* cells

Colonies of the ascidian host, Lissoclinum bistratum growing on patches of benthic macrophytes and on the leaves of seagrasses or L. patella growing on the upper surfaces of coral rubble were collected at Palau, West Caroline Islands. The animal colonies, usually found 1-3 m below surface water, were taken and promptly transported in seawater to the laboratory aboard the Japanese research vessel Sohgen-Maru. Individual colonies were cleaned of contaminants and the algal cells isolated from the host by squeezing gently by hand. The algae were then received in seawater buffered with 40 mM Tris at pH 8.4 and concentrated by centrifugation at about 60 x g for 120 s.

Measurement of carbonic anhydrase activity

For the assay of CA activity, the algae isolated from the host were suspended in 20 mM Veronal-H_2SO_4 buffer pH 8.3. The enzyme activity on the cell surface (extracellular activity) was assayed directly on such suspensions whereas total activity was assayed in homogenates disrupted by sonication. The difference between the total and extracellular activities represents the intracellular activity. When the effects of CA inhibitors, acetazolamide (AZA) and ethoxzolamide (EZA) were examined, small volumes of the compounds were added to the assay buffer prior to addition of the sample, to provide the appropriate final concentration. The assay method and expression of CA activity were the same as described previously (Dionisio et. al., 1989) with the enzyme activity units expressed on a chlorophyll basis. The concentration of chlorophyll extracted with methanol was determined according to Mackinney (1944).

Determination of photosynthetic oxygen evolution

Cells collected by centrifugation were washed twice and suspended in freshly prepared CO_2-free seawater buffered with 40 mM Tris at pH 8.4. The cell suspension (5 mL)

at a density of 10 mg chlorophyll per liter was placed in a water-jacketed cylinder equipped with a Clark-type oxygen probe. This was illuminated from one side by a projector lamp at the desired photon flux density of 250 μmol m^{-2} s^{-1}. The temperature was kept at 30 °C by water running through the water jacket and thermostat. Initially, the algal suspension was preilluminated until the endogenous carbon source was depleted as measured by cessation of oxygen evolution. The photosynthetic reaction was then started by injecting known amounts of $NaHCO_3$ solution through narrow hole in the cap of the reaction vessel. Change of oxygen concentrations in the algal suspension was monitored with a recorder connected to the oxygen probe.

Detection of CA with antiserum

For the detection of CA protein with antiserum electrophoresis of soluble protein extracts was first carried out on 12.5% (w/v) polyacrylamide gel according to Laemmli (1970) and electrotransferred to polyvinylidene diflouride filter (Bio-Rad, Richmond, Calif., USA) according to Towbin et. al., (1979). The electrotransferred proteins in the filter blot were then probed with antiserum against extracellular CA of Chlamydomonas (Dionisio-Sese et. al., 1990) and spinach chloraplastic CA. Bound CA antibodies in the filter were detected with goat anti-rabbit IgG conjugated horseradish peroxidase acting upon 3,3'-diaminobenzidine tetrahydrochloride (De Blas and Cherwinski, 1983).

RESULTS

Measurement of CA activity of Prochloron isolated from *Lissoclinum bistratum* and *L. patella* showed that both species exhibited majority (90%) of which is located on the cell surface, and only about 10% of the total CA activity is located intracellularly (Table 1). The possibility that this extracellular CA activity may be attributed to the ascidian host can be excluded since contamination by the host tissue in the cell preparation is negligible and measurement of CA activity in the animal tissue after removal of the algal cells did not show any activity.

To determine the characteristic features of CA in this microalga, the effects of the two most widely used potent sulfonamide CA inhibitors, acetazolamide (AZA) and ethoxzolamide (EZA), on CA activity of intact cells of *Prochloron* isolated from *L. patella* were examined. Sulfonamides were chosen since they were long recognized as specific high-affinity inhibitors of CA from a variety of sources (Maren, 1984). The measured I_{50} values, which are the concentration of the inhibitors required to cause 50% inhibition of activity, for the inhibition by AZA and EZA of extracellular CA from *Prochloron* are 700 μM and 300 μM, respectively (Dionisio-Sese *et. al.*, 1993).

The effect of this acetazolamide concentration on the rate of photosynthetic oxygen evolution of Prochloron at varying $NaHCO_3$ concentration was then studied. At the optimum photon flux density of 250 μmol m^{-2} s^{-1}, addition of 700 μM AZA lowered the rates

of photosynthesis under low $NaHCO_3$ concentrations (Fig. 1). As a consequence, the apparent affinity for inorganic carbon at low $NaHCO_3$ concentrations, measured as K1/2 ($NaHCO_3$), at pH 8.4 increased from 160 µM to 230 µM by acetazolamide addition. Since AZA is a membrane-impermeable sulfonamide (Moroney et. al., 1985), this result indicates that CA located on the cell surface of Prochloron increased the affinity for CO_2 in photosynthesis at low inorganic carbon concentration.

Comparison of the measured I_{50} values for inhibition of *Prochloron* CA by AZA and EZA with published data of CA from a variety of sources are shown in Table 2. It can be observed that the I_{50} values for Prochloron is very high compared to those measured for human red cell isozymes (Maren and Sanyal, 1983), the extracellular CA of unicellular chlorophyte *Chlamydomonas* (Bundy, 1986), and the intracellular CAs of the unicellular rhodophyte *Porphyridium* (Yagawa et. al., 1987a) and filamentous cyanobacterium Anabaena (Yagawa et. al., 1984). On the other hand, the unicellular cyanobacterium *Synechococcus* (Badger and Price, 1989) exhibits an I_{50} value for EZA inhibition similar to Prochloron. Likewise, Prochloron CA is similar to higher plant CA, such as spinach CA Burnell 1990) and pea CA (Atkins et al., 1972), and the intracellular CA of *Chlamydomonas* (Husic et al., 1989) in terms of sulfonamide inhibition.

To determine whether *Prochloron* CA is immunologically related to higher plant CA, like spinach CA, and not to *Chlamydomonas* extracellular CA as suggested from the sulfonamide inhibition results, immunoblot analysis was carried out using antisera against these two CAs (Fig. 2). The anti-extracellular CA antibody reacted with the 37 kiledalton (kDa) CA monomer in the soluble protein extracts from *Chlamydomonas*. It did not, however, cross-react with soluble protein extracts from spinach or Prochloron, thus confirming the inhibition results. Similarly, the anti-spinach CA antibody did not cross-react with *Chlamydomonas* CA. With *Prochloron*, however, a single immunosignal of approximately 34 kDa was observed with anti-spinach CA antibody. Since *Prochloron* CA and spinach CA exhibit almost the same sensitivity to sulfonamide, the 34 kDa band which is antigenically similar to spinach CA might be the *Prochloron* CA. This result, however, should be taken with caution since, aside from the major 26 kDa band which corresponds to the spinach CA monomer, the antibody also reacted with other proteins in the extracts, indicating the low specificity of the antiserum used.

DISCUSSION OF RESULTS

Previous reports have shown that microalgae have CA localized either on the cell surface and/or inside the cells (Aizawa and Miyachi, 1986; Tsuzuki et. al., 1984; Miyachi et. al., 1983). Among these microalgae, most chlorophytes exhibit CA activity which is predominantly associated with the cell surface (Aizawa and Miyachi 1986). In cyanobacteria, CA activity is localized inside the cells and no extracellular CA activity has been reported to date (Badger and Price 1989); Lanares et. al., 1985; Yagawa et. al., 1984). *Prochloron*, although a prokaryote, differs then from the cyanobacterial group, and bears close resemblance to chlorophytes, in exhibiting CA activity predominantly on the cell surface (Table 1).

With regards to the role of this extracellular CA in Prochloron, acetazolamide addition caused a decrease in the efficiency with which external inorganic carbon is used for photosynthesis (Fig. 1). This result is consistent with the suggested role of CA in various microalgae, that is, extracellular CA which is located either in the periplasmic space or attached to the cell wall (Kimpel et. al., 1983) functions in increasing the efficiency with which cells can access external inorganic carbon (Badger and Price, 1984; Sültemeyer et al., 1993). This involves facilitating the supply of CO_2 into the cell from HCO_3- which is the form predominant at high pH values (Miyachi et al , 1983; Tsuzuki, 1983). Thus, with the aid of extracellular CA, *Prochloron* cells have access to the large HCO_3- pool at alkaline pH values in seawater via indirect acquisition of HCO_3-. As to the role of intracellular CA, it is thought to be involved in increasing the steady-state flux of CO_2 within the cell thereby enhancing the supply of CO_2 to RuBisCO (Tsuzuki, 1986; Badger et al., 1985; Imamura et al., 1981).

Though CA in both *Synechococcus* and *Anabaena* is assumed to be localized within the RuBisCO-containing carboxysomes (Badger and Price, 1989), these two cyanobacteria exhibit highly different I_{50} values in terms of CA inhibition by sulfonamides; the former is less sensitive than the latter (Table 2). It is interesting to note that on the basis of 16S ribosomal RNA sequence data, these species are two of the most highly divergent cyanobacteria known (Giovannoni et al., 1988). Since inhibitors like sulfonamides are thought to bind near the active site of the enzyme (Maren and Sanyal 1983), the difference in sensitivity to sulfonamides may reflect differences at or near the active site of these enzymes. Another microalga whose CA activity is sensitive to sulfonamide, the rhodopyte Porphyridium has CA localized mainly in the chloroplast (Yagawa et al., 1987b). CA from higher plants, on the other hand, are generally considered to be relatively resistant to sulfonamides. Although there is evidence that cytoplasmic isozymes of CA are present in leaves of some plants, the majority of leaf CA activity in spinach is localized in the chloroplast (Tsuzuki et al., 1985; Werdan and Heldt, 1972), specifically in the stroma (Poincelot, 1972). The presence of transit peptide in cDNA coding for pea CA suggest that CA activity in pea also resides within the chloroplast (Majeau and Coleman, 1991). With regards to the sulfonamide-resistant intracellular CA of Chlamydomonas, although a cytoplasmic form of enzyme exists, it was suggested that the observed I_{50} values probably correspond to the form of CA within the chloroplast (Husic et al., 1989). Using immunological techniques, a 45 kilodalton polypeptide immunoreactive with the anti-spinach CA antiserum was detected in the chloroplast stromal fraction (Husic et al., 1989). Recently, there was a report that CA associated with the chloroplast in *Chlamydomonas* is insoluble, suggesting that it is membrane-bound (Sültemeyer, 1990). In another green alga Chlorella, Pronina and Semenenko (1984) reported an insoluble membrane-bound CA which is associated with the chloroplast membranes.

Since *Prochloron* exhibits appressed thylakoid membranes containing chlorophylls a and b characteristics of the chloroplasts of green algae and higher plants, some workers in the field of endosymbiosis favour the idea that the green algal chloroplasts may have arisen by the uptake of *Prochloron* as symbionts (Whatley et al., 1979). Comparison of the

sequences of psbA genes, which encode the photosystem II thylakoid protein D1, from a related free-living, filamentous prochlorophyte, *Prochlorothrix*, with those reported for cyanobacteria, a green alga, a liverwort and several higher plants places the prochlorophytes closer also to green plant chloroplasts than cynobacteria (Morden and Golder, 1989). On the other hand, sequence comparison of the genes encoding the 16S ribosomal RNA (Turner et al., 1989; Seewaldt and Stackebrandt, 1982), the large and small subunits of RuBisCo (Morden and Golden, 1991) and subunit of DNA-dependent RNA polymerase (Palenik and Haselkorn, 1992) places the prochlorophytes more closely related to cyanobacteria than to the green plastid lineage. Recently, on the basis of 16S ribosomal RNA data, it was suggested that prochlorophytes are polyphyletic within the cyanobacteria radiation, and not specifically related to chloroplasts (Urbach et al., 1992).

The results presented in this paper showed that in terms of CA inhibition by sulfonamide, *Prochloron* is similar to both the unicellular cyanobacteria and to chloroplasts of green algae and higher plants. On this aspect, then, *Prochloron* shares characters with both cyanobacteria and green chloroplasts, suggesting a possible link between the cyanobacteria and chlorophytes. Since sulfonamide inhibition of CA is attributed to its binding with the active site of the enzyme, it may be that the structure of active site in CAs from *Prochloron*, *Synechococcus*, and chloroplasts of *Chlamydomonas*, spinach and pea are quite similar to each other. When Western blot analysis was carried out to determine whether soluble protein extracts of *Prochloron* cross-react with anti-spinach CA antibody, a single immunosignal of approximately 34 kDa was observed (Fig. 2). However, concluding that this was *Prochloron* CA would be difficult since the antibody also reacted with some other proteins in the spinach soluble extracts.

At present, cDNAs coding for the spinach chloroplastic CA (Burnell et al., 1990), pea chloroplastic CA (Majeau and Coleman, 1991) and *Chlamydomonas* extracellular CAs (Fukuzawa et al., 1990) have been isolated and characterized. No significant sequence similarity has been observed between these CAs (Fukuzawa et al., 1991). More recently, a putative CA gene showing significant sequence similarity to spinach and pea chloroplastic CA but not to *Chlamydomonas* extracellular CA has been identified in *Synechococcus* (Fukuzawa et al., 1992). Spinach CA and pea CA in the same paper was then suggested to be prokaryotic in nature whereas the Chlamydomonas extracellular CA, which shares sequence similarity with mammalian CAs (see also Fukuzawa et al., 1991; 1990) was suggested to be a eukaryotic type. Since *Prochloron* CA exhibits I_{50} values similar to spinach CA and *Synechococcus* CA but highly different from *Chlamydomonas* extracellular CA or mammalian CA, it would of the interest to determine whether Prochloron CA exhibits sequence similarity with the prokaryote-type CAs.

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Ascidian host	CA Activity (U . mg chl^{-1})		
	Extracellular	Intracellular	Total
Lissoclinum bistratum	6.21	0.56	6.77
Lissoclinum patella	5.35	0.57	5.92

Table 1. Carbonic anhydrase activity of Prochloron cells isolated from ascidian host.

Enzyme and source	I_{50} (μM)		Reference
	Acetazolamide	Ethoxzolamide	
Human Erythrocyte CA I	0.2	0.002	Maren and Sanyal, 1983
Human Erythrocyte CA II	0.01	0.002	Maren and Sanyal, 1983
Chlamydomonas Extracellular CA	0.002	0.005	Bundy, 1986
Chlamydomonas Intracellular CA	300	20	Husic et al., 1989
Spinach CA	100	1	Burnell, 1990
Prochloron CA	450	5	Atkins et al , 1972
Synechococcus CA	700	300	Dionisio-Sese et al., 1993
Anabaena CA	-	50	Bedger and Price, 1989
Porphyridium CA	0.3	0.003	Yagawa et al., 1984
	0.09	0.1	Yagawa et al., 1987a

Table 2. Comparison of I_{50} values for acetazolamide and ethoxyzolamide inhibition of carbonic anhydrase from Prochloron, human erythrocytes, spinach, pea and various microalgal species.

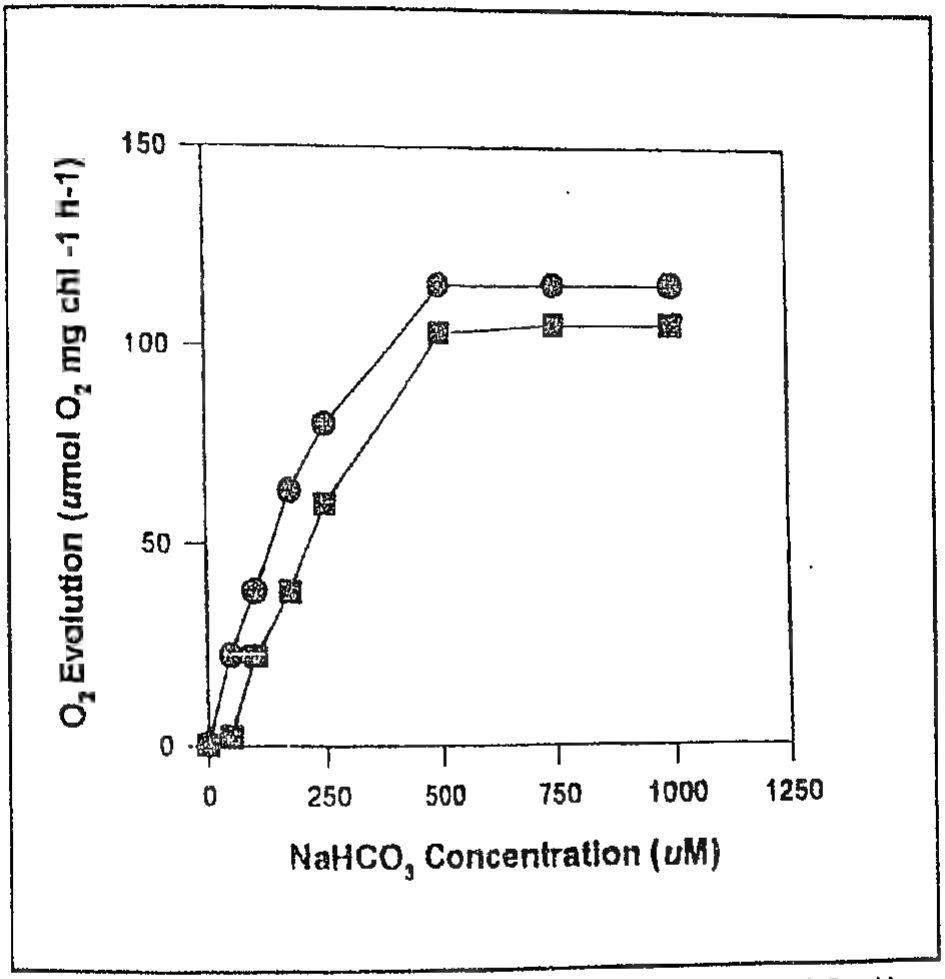

Figure 1. Effect of CA inhibitor acetazolamide on the photosynthetic rates of Prochloron at varying concentration of $NaHCO_3$. Rate of oxygen evolution determined in the absence (●) and presence (■) of 700 µM acetazolamide.

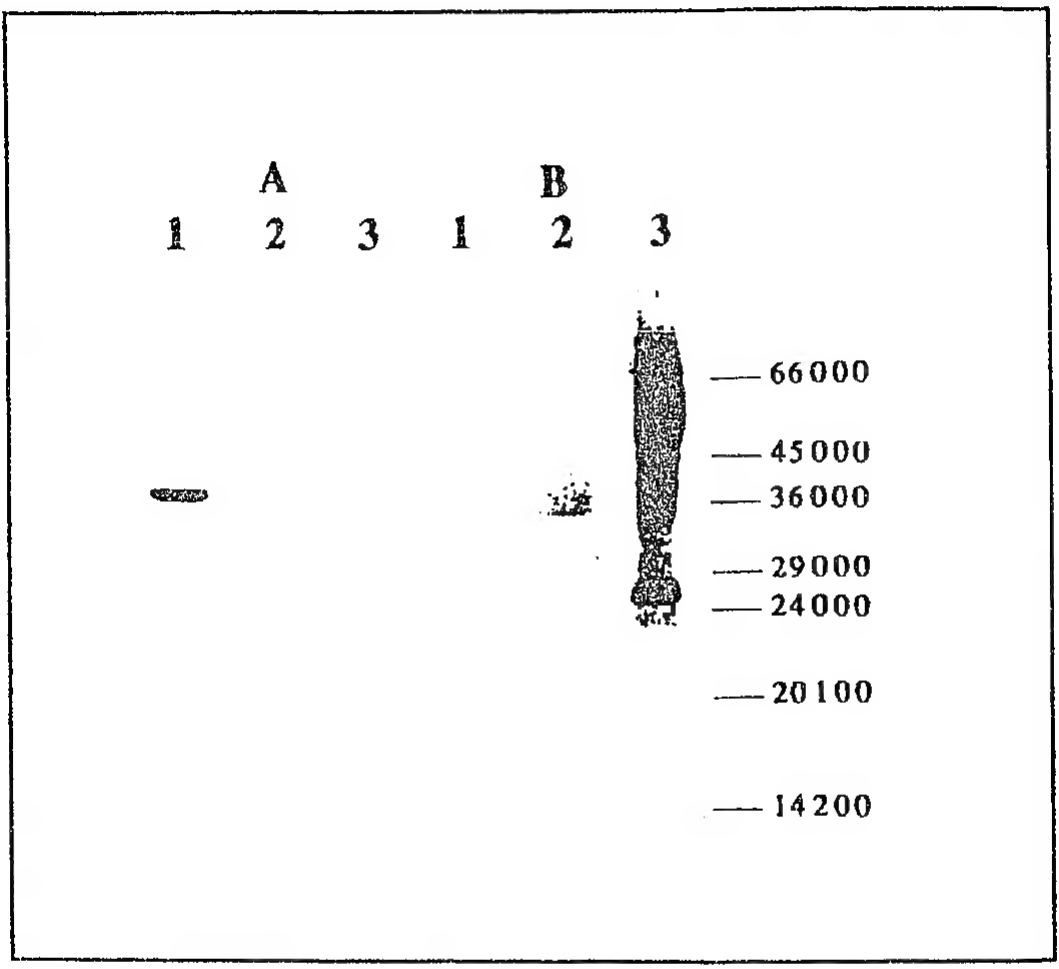

Figure 2. Immunoblots of soluble protein extracts from Chlamydomonas (1), Prochloron (2) and spinach (3) probed with antibodies against extracellular CA of Chlamydomonas (A) and spinach intracellular CA (B). Molecular weight markers indicated to the right in the figure are in dalton units.

Guide to Authors

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Mass	
kilogram	kg
gram	g
ton (metric ton)	t
milligram	mg
Time (same units used in both Metric and English System)	
day	d
hour	h
minute	min
second	s
Amount of substance	
mode	mole
Temperature	
degree celsius	^oC